PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Robert HALEY *et al.*	Group Art Unit: 1635
Serial No.: 10/039,171	Examiner: B. Whiteman
Filed: January 3, 2002	Atty. Dkt. No.: UTSD:749US
For: COMPOSITIONS AND METHODS FOR THE DIAGNOSIS AND TREATMENT OF ORGANOPHOSPHATE TOXICITY	Confirmation No.: 7156

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Steven L. Highlander

APPEAL BRIEF

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APPEAL BRIEF

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-01450

Dear Sir:

This Appeal Brief is filed in response to the Office Action mailed on October 2, 2008. Also included herewith is a Notice of Appeal under 37 C.F.R. §41.31. Should any fees be due, the Commissioner is authorized to debit Fulbright & Jaworski L.L.P. Deposit Acct. No. 50-1212/UTSD:749US/SLH.

I. Real Party In Interest

The real party in interest is the assignee, the Board of Regents, University of Texas System, Austin, TX.

II. Related Appeals and Interferences

There are no related appeals or interferences.

III. Status of the Claims

Claims 1-35 were filed with the original application, and claims 36-43 were added during prosecution. Claims 6-8 and 26-35 have been canceled. Thus, claims 1-5, 9-25 and 36-43 are rejected and stand appealed. Claims A copy of the appealed claims is attached as Appendix A.

IV. Status of the Amendments

No amendments were offered following mailing of the final Office Action.

V. Summary of the Claimed Subject Matter

Independent claim 1 sets forth a method of protecting a cell from organophosphate toxin comprising (a) identifying a cell at risk of exposure or exposed to an organophosphate toxin; (b) providing an expression cassette comprising a promoter active in said cell and a gene encoding PON1 under the control of said promoter; and (c) transferring said expression cassette into said cell under conditions permitting expression of PON1; wherein said expression cassette expresses PON1 in said cell, providing protection from said organophosphate toxin. This claim is supported at page 4, lines 5-10 of the specification.

Claim 21, the other independent claim, sets forth a method of protecting a subject from an organophosphate toxin comprising (a) identifying a subject at risk of exposure or exposed to an organophosphate toxin; (b) providing an expression cassette comprising a promoter active in cells of said subject, a gene encoding PON1 under the control of said promoter; and (c) administering to said subject said expression cassette under conditions permitting expression of PON1; wherein said expression cassette expresses PON1 in said cell, providing protection from said organophosphate toxin. This claim is supported at page 4, lines 19-23 of the specification.

VI. Grounds of Rejection to be Reviewed on Appeal

1. Are claims 1-5, 10-13, 19-25, 37-39 and 43 obvious under 35 U.S.C. §103 over Radtke (Exhibit 1) taken with Li *et al.* (Exhibit 2), Davies *et al.* (Exhibit 3), Adkins *et al.* (Exhibit 5) and Humbert *et al.* (Exhibit 6)?
2. Are claims 29 and 43 obvious under 35 U.S.C. §103 over Radtke, Li *et al.*, Davies *et al.*, Adkins *et al.* (Exhibit 5), Humbert *et al.* (Exhibit 6) and Scheffler (Exhibit 4)?

VII. Argument

A. Standard of Review

Findings of fact and conclusions of law by the U.S. Patent and Trademark Office must be made in accordance with the Administrative Procedure Act, 5 U.S.C. §706(A), (E), 1994. *Dickinson v. Zurko*, 527 U.S. 150, 158 (1999). Moreover, the Federal Circuit has held that findings of fact by the Board of Patent Appeals and Interferences must be supported by "substantial evidence" within the record. *In re Gartside*, 203 F.3d 1305, 1315 (Fed. Cir. 2000). In *In re Gartside*, the Federal Circuit stated that "the 'substantial evidence' standard asks whether a reasonable fact finder could have arrived at the agency's decision." *Id.* at 1312. Accordingly, it necessarily follows that an examiner's position on appeal must be supported by "substantial evidence" within the record in order to be upheld by the Board of Patent Appeals and Interferences.

B. Rejections Under 35 U.S.C. §103

i. Radtke, Li, Adkins, Humbert and Davies

Claims 1-5, 10-13, 19-25, 37-39 and 43 stand rejected over Radtke (U.S. Patent 6,521,226) in view of Li *et al.* (1995), Davies *et al.* (1996), Adkins *et al.* (1993) and Humbert *et al.* (1993). According to the examiner, Radtke provides all the necessary disclosure for recombinant expression of PON1, including PON1 Q and R, in humans, but fails to provide the motivation to do so in a subject exposed or at risk of OP toxicity. Li *et al.* is said to provide the missing motivation to use PON1 expression vectors such as a treatment or prevention of OP toxicity given that the reference allegedly teaches that "paraoxonase protects animals against organophosphate toxicity." Davies *et al.* is cited as representative of knowledge in the field that "the main determinant of susceptibility to organophosphate poisoning is the activity level of PON 1 enzymes ... across many species including humans." Adkins *et al.* and Humbert *et al.* are merely cited as teaching that PON1 type R hydrolyzes paraoxon rapidly. Appellants traverse.

To determine obviousness, the examiner must follow a strict set of guidelines from which he or she cannot deviate:

> To reach a proper determination under 35 U.S.C. 103, the examiner must step backward in time and into the shoes worn by the hypothetical "person of ordinary skill in the art" when the invention was unknown and just before it was made. In view of all factual information, the examiner must then make a determination whether the claimed invention "as a whole" would have been obvious at that time to that person. Knowledge of applicant's disclosure must be put aside in reaching this determination, yet kept in mind in order to determine the "differences," conduct the search and evaluate the "subject matter as a whole" of the invention. The tendency to resort to "hindsight" based upon applicant's disclosure is often difficult to avoid due to the very nature of the examination process. However, impermissible hindsight must be avoided and the legal conclusion must be reached on the basis of the facts gleaned from the prior art....
>
> To establish a *prima facie* case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. ***Second, there must be a reasonable expectation of success.*** Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations. ***The teaching or suggestion to make the***

> ***claimed combination and the reasonable expectation of success must both be found in the prior art***, and not based on applicant's disclosure. *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991). See MPEP § 2143 - § 2143.03 for decisions pertinent to each of these criteria ....

MPEP §2142 (emphasis added). Here, appellants submit that the examiner cannot point to ***either*** an adequate motivation in the cited art or the field in general to support the combination of references, nor is there any indication that one of skill in the art would have found anything like a ***reasonable*** likelihood of success in practicing the invention as now claimed.

At the outset, appellants readily acknowledge that Radke does indeed disclose information needed to provide for the recombinant expression of PON1 Q and R. However, as has been pointed out repeatedly, and acknowledged by the examiner, this reference provides ***no*** motivation to use it for treating or preventing OP toxicity. In attempting to remedy this clear defect, the examiner previously attempted rely on Li *et al.* and Davies *et al.*, and now turns additionally to Adkins *et al.* and Humbert *et al.* As will be shown, these references are limited technically, and thus do not support the present rejection as argued by the examiner.

First, it is important to note that Li *et al.* isolated "PON" from rabbits, not PON1, and thus it is highly uncertain what this composition contained. They certainly report no effort to characterize the content of this composition. What ***is*** certain is that PON1 L and PON1 M were present, as well as PON1 Q and PON1 R, all in unknown distributions. Indeed, it is also quite possible that PON2 and PON3 enzymes were present. So, from the teachings of Li, one of skill in the art could not possibly know ***which*** element of the composition was protecting the animals from chlorpyrifos toxicity. So, even with Radtke and Li combined, one still cannot link PON1 Q and/or R with protection from OP toxicity.

Next, turning to Davies *et al.*, this article does suggest a role for PON1 Q and R in protection from OP toxicity and differential potency of the PON1 Q and R isoenzymes in

hydrolyzing different OPs. However, Davies' demonstration of substrate specificity for PON1 Q and R on serum samples tested *in vitro* was not sufficient to demonstrate that ***boosting*** PON1 Q and R isoenzyme concentrations *in vivo* would successfully protect against OP poisoning. The experiment of Davies *et al.* simply tested the hydrolytic activity of 92 serum samples of hispanic subjects to several OP chemicals *in vitro*. They did not test whether boosting the PON1 Q and R activity levels ***above*** what the subjects normally had would ***increase*** the protective effect. A genetic therapy depends on more factors than the gene activity for its success. For example, PON1 Q and R isoenzymes are bound to the high density lipoprotein (HDL) particle *in vivo*, and it was possible that the concentrations of HDL particles (PON1 binding capacity), or other physiologic factors, might have limited the hydrolytic effectiveness of the increases in production of the PON1 Q and R isoenzymes by the genetic therapy device. Many other potential influences could have limited the effects or increased the toxicity of boosting PON1 Q and R activity levels *in vivo*. None of these limitations could be discovered by simply measuring the differential hydrolysis rates of PON1 isoenzymes on serum samples *in vitro*.

The newly cited Adkins *et al.* and Humbert *et al.* references are argued as providing nothing more than point to PON1 R as being more active in hydrolyzing paraoxon. Even if true, this cannot address the fundamental deficiency, set out above, that entirety of the cited references fail to not indicate what will happen when one ***boosts*** the levels of PON1 R (or PON1 Q).

Finally, in contrast to the cited art, appellants were the first to demonstrate that substrate specificity was successfully produced by boosting PON1 Q and R levels *in vivo*, removing the uncertainty over the many possibly perturbing influences and demonstrating that no apparent toxic effects limited its usefulness. Without this information, one skilled in the art could not possibly have assured that a genetic therapy for OP toxicity could be successfully produced and

offered. Likewise, Davies *et al.* state that "***We also show that the effect of the PON1 polymorphism is reversed for the hydrolysis of diazoxon, soman and especially sarin, thus changing the view of which PON1 isoform is considered to be protective***." Abstract (emphasis added). Thus, it appears that Davies considered substrate specificity to be important, but determining such *in vitro* is not sufficient to predict what happens *in vivo*. This unpredictability is highlighted by Radtke, who taught that PON1 Q was the only important PON1 enzyme, albeit for atherosclerosis and not OP toxicity – an assertion now known to be false in the context of protecting against toxicity by some important OPs. Because the present inventors were the first to demonstrate the *in vivo* protective effects of PON1 Q/R recombinant therapy, including that PON1 R provided much better protection from chlorpyrifos than PON1 Q, they were the first to enable such treatments. The recently added citations of Adkins and Humbert do nothing to address this defect.

In sum, Radtke showed how to use PON1 Q and R in genetic therapy but held that only PON1 R was useful, Li showed that infusing an unspecified "PON" mixture imparts protection from OP's *in vivo*, Davies showed that varying PON1 Q and R confer different levels of protection to different OP's, but these were innate levels that were not boosted, and Adkins and Humbert do no more than provide limited information on the relative activities of PON1 Q and R on a single agent - paraoxonase. However, none of these papers address the question of whether ***boosting both PON1 Q or R*** will protect differentially from OP exposures *in vivo*. There simply were too many unknowns that had not been addressed, any of which could have made the concept fail. Only by performing the experiment *in vivo* and showing that it worked could one claim to have possession of the reasonable predictability needed for obviousness, and that showing is missing from the prior art.

The examiner has attempted to counter these very strong arguments, not by any scientific rebuttal, but by taking refuge behind three legal principles – first, that appellants have no "evidence" of unpredictability; second, that appellants are "arguing the references separately"; and third, that the recent *KSR* decision provides some sort of carte blanche for trivializing the motivation requirement of §103 rejections. As will be explained, ***all of these arguments are false***.

Addressing the evidence of unpredictability, appellants continue to believe that the U.S. PTO is estopped in this case from taking the position that gene therapy is a predictable art (if it were ***not*** taking this position, there would be no basis for challenging appellants' statements in this regard). Regardless, it is not necessary to discuss the general position of the PTO on gene therapy here for the simple reason that the present examiner has already gone on record in this prosecution that gene therapy ***is***, in fact, unpredictable: "Thus, at the time the application was filed, the state of the art for gene therapy was considered highly unpredictable." Office Action of August 23, 2005, page 6; Office Action of May 17, 2006, page 5. The examiner cannot now argue there is only attorney argument to support this issue.

The examiner argues that because the rejection has been withdrawn in view of appellants' "argument," prior comments on the record are somehow irrelevant. Nothing could be further from the truth. Appellants overcame the enablement rejection not by arguing in favor of predictability, but by showing that present invention worked in an art accepted animal model. Thus, the ***factual*** statement, made by and stood behind the by the PTO, that gene therapy is unpredictable, cannot simply be "waived off" when it no longer suits the PTO's purposes.

Turning to the question of "arguing references separately," appellants submit that this is not what the record reveals. Rather, what appellants have done is to point out specific defects in

each reference that undercut the examiner's attempted extrapolations. The skilled artisan would have read each of these references at the time the present invention was made and drawn from them only that which the science therein supports. Thus, it is only fair to for appellants to filter the teachings of these references in the same way that the skilled artisan would before combining their teachings. To do otherwise is to ignore that basic tenets of science that guide and direct the skilled artisan. As such, the arguments regarding examining the references individually simply do not hold any merit.

Lastly, the examiner has improperly cited *KSR* as supporting the combination of five ***fundamentally limited references***. While that case did indeed address the question of how explicit a suggestion in the art must be in order to support combining of various art, and thus whether motivation to combine can be established from more general teachings in the field, ***it did not change decades of case law requiring that motivation must be present***. Indeed, the Supreme Court stated that such an obvious to try rationale ***may*** support a conclusion that a claim would have been obvious where one skilled in the art is choosing from ***a finite number of identified, predictable solutions, with a reasonable expectation of success***. *KSR International Co. v. Teleflex Inc.*, 127 S. Ct, 1727, 1742, 82 USPQ2d 1385, 1397 (2007). However, where obvious to try means that one would have to "vary all parameters or try each of numerous possible choices until one possibly arrived at a successful result, where the prior art gave either no indication of which parameters were critical or no direction as to which of many possible choices is likely to be successful ...," that would not satisfy §103. *In re O'Farrell*, 853 F.2d 894, 903, 7 USPQ2d 1673, 1681 (Fed. Cir. 1988). Similarly, where obvious to try means the exploring of "a new technology or general approach that seemed to be a promising field of experimentation, where the prior art gave only general guidance as to the particular form of the

claimed invention or how to achieve it ...," that too would be insufficient. *Id.* Here, there is clearly no "finite number of identified, predictable solutions" from which the skilled artisan could choose, as the examiner's own earlier admission recognized, not to mention the failure of Radtke to exploit this allegedly "obvious" aspect of PON1 gene therapy.

Thus, though the prior art may have pointed in the general direction of using PON1 to treat or protect from OP toxicity, the art relied upon is technically limited, and the entire endeavor is acknowledged to be far too speculative for those of skill in the art to consider it in any way predictable or straightforward. As such, no *prima facie* case can be deemed to have been made out. Reversal of the rejection is therefore again respectfully requested.

ii. Radtke, Li, Davies, Adkins, Humbert and Scheffler

Claims 1, 9, 14-16, 21, 36 and 40-42 are rejected as obvious over Radtke, Li *et al.*, Davies *et al.*, Adkins *et al.*, and Humbert *et al.* in view of Scheffler (U.S. Patent 5,721,118) under §103. The first three references are cited as above, and Scheffler is cited as teaching poly-A sequences and various promoters. Appellants traverse.

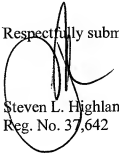
As discussed above, Radtke, Li, Davies, Adkins and Humbert do not render the present invention obvious. Scheffler, providing only structural elements for expression vectors, does not cure this defect, and as such, this rejection is improper as well.

Thus, for the reasons set forth above, reversal of this rejection also is respectfully requested.

C. Conclusion

In light of the foregoing, appellant respectfully submits that all pending claims are non-obvious under 35 U.S.C. §103. Therefore, it is respectfully requested that the Board reverse each of the pending rejections.

Respectfully submitted,



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Date: December 1, 2008

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VIII. APPENDIX A – APPEALED CLAIMS

1. A method of protecting a cell from organophosphate toxin comprising:

   (a) identifying a cell at risk of exposure or exposed to an organophosphate toxin;

   (b) providing an expression cassette comprising a promoter active in said cell and a gene encoding PON1 under the control of said promoter; and

   (c) transferring said expression cassette into said cell under conditions permitting expression of PON1;

   wherein said expression cassette expresses PON1 in said cell, providing protection from said organophosphate toxin.

2. The method of claim 1, wherein PON1 is PON1 type Q.

3. The method of claim 1, wherein PON1 is PON1 type R.

4. The method of claim 1, wherein said cell expresses PON1 type Q.

5. The method of claim 1, wherein said cell expresses PON1 type R.

9. The method of claim 1, wherein said expression cassette further comprises a polyadenylation signal.

10. The method of claim 1, wherein said expression cassette is further comprised within a vector.

11. The method of claim 10, wherein said vector is a viral vector.

12. The method of claim 11, wherein said viral vector is a herpesviral vector, a retroviral vector, an adenoviral vector, an adeno-associated viral vector, a polyoma viral vector, and a vaccinia viral vector.

13. The method of claim 11, wherein said viral vector is an adenoviral vector.

14. The method of claim 1, wherein said promoter is a constitutive promoter.

15. The method of claim 1, wherein said promoter is an inducible promoter.

16. The method of claim 1, wherein said promoter is a tissue specific promoter.

17. The method of claim 4, wherein said expression cassette increases PON1 type Q expression by about 10-fold.

18. The method of claim 5, wherein said expression cassette increases PON1 type R expression by about 10-fold.

19. The method of claim 1, wherein said cell is a liver cell.

20. The method of claim 1, wherein said cell expresses low levels of PON1 type Q or R as compared to the general population.

21. A method of protecting a subject from an organophosphate toxin comprising:

(a) identifying a subject at risk of exposure or exposed to an organophosphate toxin;

(b) providing an expression cassette comprising

(i) a promoter active in cells of said subject,

(ii) a gene encoding PON1 under the control of said promoter; and

(c) administering to said subject said expression cassette under conditions permitting expression of PON1;

wherein said expression cassette expresses PON1 in said cell, providing protection from said organophosphate toxin.

22. The method of claim 21, wherein PON1 is PON1 type Q.

23. The method of claim 21, wherein PON1 is PON1 type R.

24. The method of claim 38, wherein said viral vector is a herpesviral vector, a retroviral vector, an adenoviral vector, an adeno-associated viral vector, a polyoma viral vector, and a vaccinia viral vector.

25. The method of claim 21, wherein administering comprises intravenously or intraarterially.

36. The method of claim 21, wherein said expression cassette further comprises a polyadenylation signal.

37. The method of claim 21, wherein said expression cassette is further comprised within a vector.

38. The method of claim 37, wherein said vector is a viral vector.

39. The method of claim 38, wherein said viral vector is an adenoviral vector.

40. The method of claim 21, wherein said promoter is a constitutive promoter.

41. The method of claim 21, wherein said promoter is an inducible promoter.

42. The method of claim 21, wherein said promoter is a tissue specific promoter.

43. The method of claim 21, wherein cells of said subject express low levels of PON1 type Q or R as compared to the general population.

IX. APPENDIX B – EVIDENCE CITED

Exhibit 1 – Radtke, U.S. Patent 6,521,226

Exhibit 2 – Li *et al.* (1995)

Exhibit 3 – Davies *et al.* (1996)

Exhibit 4 – Scheffler, U.S. Patent 5,721,118

Exhibit 5 – Adkins *et al.* (1993)

Exhibit 6 – Humbert *et al.* (1993)

X. APPENDIX C – RELATED PROCEEDINGS

None

EXHIBIT 1

US 6521226B1

(12) **United States Patent**
Radtke

(10) **Patent No.: US 6,521,226 B1**
(45) **Date of Patent: Feb. 18, 2003**

(54) **METHOD OF USING PON-1 TO DECREASE ATHEROMA FORMATION**

(75) Inventor: **Klaus-Peter Radtke**, Apex, NC (US)

(73) Assignee: **Bayer Corporation**, Pittsburgh, PA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **10/105,640**

(22) Filed: **Mar. 25, 2002**

Related U.S. Application Data

(62) Division of application No. 09/199,672, filed on Nov. 25, 1998, now Pat. No. 6,391,298.

(51) **Int. Cl.**[7] A61K 38/46; C12N 9/16; C12N 1/20; C07K 1/00; C07H 21/04

(52) **U.S. Cl.** 424/94.6; 435/196; 435/252.3; 435/320.1; 530/350; 530/387.1; 536/23.2

(58) **Field of Search** 424/94.6; 435/196, 435/252.3, 320.1; 530/350, 387.1; 536/23.2

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,629,193 A 5/1997 Hudson et al. 435/325

FOREIGN PATENT DOCUMENTS

WO 96/01322 1/1996

OTHER PUBLICATIONS

Adkins, S., et al., "Molecular Basis for the Plymorphic Forms of Human Serum Paraoxonase/Arylesterase: Glutamine or Arginine at Position 191, for the Respective A or B Allozymes", *Am. J. Hum. Genet. 52*: 598–608 (1993).

Aviram, M., et. al.,, "Paraoxonase Inhibits High-density Lipoprotein Oxidation and Preserves its Functions. A Possible Peroxidative Role for Paraoxonase", *J. Clin. Invest.101(8)*: 1581–1590 (Apr. 1998.)

Blatter, M–C., et al., "Identificatin of a distinct human high–density lipoprotein subspecies defined by a lipoprotein–asociated protein, K–45. Identity of K–45 with paraoxonase", *Eur. J. Biochem. 211*: 871–879 (1993).

Blatter Garin, M–C., et al., "Paraoxonase Polymorphism Met–Leu54 is Associated with Modified Serum Concentrations of the Enzyme", *J. Clin. Invest. 99(1)*: 62–66 (Jan. 1997).

Chemnitius, J., et al., "Organophosphate Detoxicating Hydrolases in Different Vertebrate Species", *Comp. Biochem. Physiol 76C*: 85–93 (1983).

Eckerson, H. W., et al., "The Human Serum Paraoxonase/Arylesterase Polymorphism", *Am. J. Hum. Genet. 35*: 1126–1138 (1983).

Ikeda, Y., et al., "Serum Paraoxonase Activity and its Relationship to Diabetic Complications in Patients with Non–Insulin–Dependent Diabetes Mellitus", *Metabolism 47(5)*: 598–602 (May 1998).

Kelso, G. J., et al., "Apolipoprotein J is Associated with Paraoxonase in Human Plasma", *Biochemistry 33*: 832–839 (1994).

Leviev, I., et al., "Two Alleles of the Human Paraoxonase Gene Produce Different Amounts of mRNA. An Explanation for Differences in Serum Concentrations of Paraoxonase Associated with the (Leu–Met54) Polymorphism", *Arteriosclerosis, Thrombosis, and Vascular Biology 17(11)*:2935–2939 (Nov. 1997).

Li, W. F., et al., "Paraoxonase Protects Against Chlorpyrifos Toxicity in Mice", *Toxicology Letters 76*: 219–226 (1995).

Li, W., et al., "Serum Paraoxonase Status: A Major Factor in Determining Resistance to Organophosphates", *J. Toxicol0gy and Environmental Health 40*: 337–346 (1993).

Lorentz, K., et al., "Arylesterase in Serum: Elaboration and Clinical Application of a Fixed–Incubation Method", *Clin. Chem. 25(10)*: 1714–1720 (1979).

Losch. H. et al., "Die Enzymatische Entigiftung von Phosphororganischen Insektiziden und Nervengasen in Primaten", *Arzneim,–Forsch./Drug Res. 32(II)*, Nr. 12: 1523–1529 (1982).

Mackness, B., et al., "Human Serum Paraoxonase", *Gen. Pharmacol. 31(3)*: 329–336 (1998).

Mackness, M. I., et al., "Paraoxonase and coronary heart disease", *Curr. Opin. Lipidol. 9(4)*:319–324 (1998).

Mackness, M. I., et al., "Presence of parazoxonase in human interstitial fluid", *Febs Letters 416*: 377–380 (1997).

Mackness, M. I., et al., "HDL, its enzymes and its potential to influence lipid peroxidation", *Atherosclerosis 115*: 243–253 (1995).

Mackness, M. I., et al., "Paraoxonase: Another Factor of NIDDM Cardiovascular Disease", *The Lancet*, 346: 856 (Sep. 30, 1995).

Mackness, M. I., et al., "Protection of low–density lipoprotein against oxidative modifcation by high–density lipoprotein associated paraoxonase", *Atherosclerosis 104*: 129–135 (1993).

Mackness, M. I., et al., "Is Paraoxonase Related to Atherosclerosis", *Chem. Biol. Interactions 87*: 161–171 (1993).

Mackness, M. I., et al., "Serum paraoxonase activity in familial hypercholesterolaemia and insulin–dependent diabetes mellitus", *Atherosclerosis 86*: 193–199 (1991).

(List continued on next page.)

Primary Examiner—Tekchand Saidha
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(57) **ABSTRACT**

The invention is directed to a method of decreasing atheroma formation in a mammal comprising administering a pharmaceutically effective amount of PON-1 or its functional equivalent to a patient in need thereof. Also included herein are pharmaceutical compositions, and a method for diagnosing predisposition to hypercholesterolemia by assessing the level of native circulating PON-1.

10 Claims, 4 Drawing Sheets-

OTHER PUBLICATIONS

Mackness, M. I., et al., "Paraoxonase Prevents Accumulation of Lupoprotein of Lipoperoxides in Low–density Lipoprotein", *Febs Letters 286(1–2)*: 152–154 (Jul. 1991).

Main, A. R., "The Role of A–Esterase in the Acute Toxicity of Paraoxon, Tepp, and Parathion", *Can. J. Biochem. Physiol. 34*: 197–215 (1956).

Masson, P., et al., "Enzymes hydrolyzing organophsphates as potential catalytic scavengers against organophosphate poisoning", *J. Physiology (Paris)* 92: 357–362 (1998).

Mochizuki, H., et al., "Human PON2 gene at 7q21.3: cloning, multiple mRNA forms, and missense polymorphisms in the coding sequence", *Gene 213*: 149–157 (1998).

Navab, M., et al., "Mildly Oxidized LDL Induces and Increased ApolipoproteinJ/Paraoxonase Ratio", *J. Clin. Invest. 99(8)*: 2005–2019 (Apr. 1997).

Parthasarathy, S., et al., High–density lipoprotein inhibits the oxidative modification of low–density lipoprotein, *Biochimica et Biophysica Acta 1044*: 275–183 (1990).

Ruiz, J., et al., "Paraoxonase/Arylesterase Gene (Met–Leu55) Polymorphism is Independently Associated with Coronary Heart Disease in NIDDM", *Complications, Macrovascular I*, pp. 65A, #233.

Ruiz, J., et al., "Paraoxonase Gene Mutation (Gln–Arg19) in Coronary Heart Disease", *Complications, Macrovascular*, pp. 35A, #127.

Ruiz J., et al., "Gln–Arg 192 polymorphism of paraoxonase and coronary heart disease in type 2 diabetes", *The Lancet*, 346: 869–872 (Sep. 30, 1995).

Sanghera, D. K., et al., "Genetic Polymorphism of Paraoxonase and the Risk of Coronary Heart Disease", *Arterioscelrosis, Thrombosis, and Vascular Biology 17(6)*: 1067–1073 (Jun. 1997).

Schiavon, R., et al., "Serum paraoxonase activity is decreased in uremic patients", *Clinica Chimica Acta 247*: 71–80 (1996).

Serrato, M., et al., "A Variant of Human Paraoxonase/Arylesterase (HUMPONA) Gene is a Risk Factor for Coronary Artery Disease", *J. Clin. Invest. 96*: 3005–3008 (Dec. 1995).

Shih, D. M., et al., "Mice lacking serum paroxonase are susceptible to organophosphate toxicity and atherosclerosis", *Nature 394*: 284–287 (Jul. 1998).

Shih, D. M., et al., "Genetic–Dietary Regulation of Serum Paraoxonase Expression and its Role in Atherogenesis in a Mouse Model", *J. Clin. Invest. 97(7)*: 1630–1639 (Apr. 1996).

Szabo, I., et al., "Is Paraoxon Hydrolytic Activity in Serum Predictive of Myocardial Infarction?", *Clin. Chem. 33(5)*: 742–743 (May 1987).

Van Lenten, B. J., et al., "Anti–inflammatory HDL Becomes Pro–inflammatory during the Acute Phase Response. Loss of Protective Effect of HDL against LDL Oxidation in Aortic Wall Cell Cocultures", *J. Clin. Invest. 96*: 2758–2767 (Dec. 1995).

Watson, A. D., et al., "Protective Effect of High Density Lipoprotein Associated Paraoxonase. Inhibition of the Biological Activity of Minimally Oxidized Low Density Lipoprotein", *J. Clin. Invest. 96*: 2882–2891 (Dec. 1995).

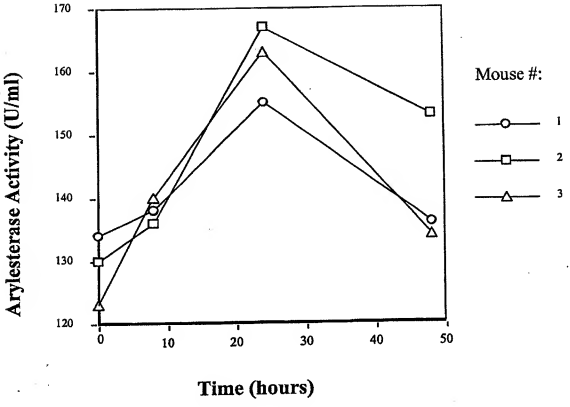
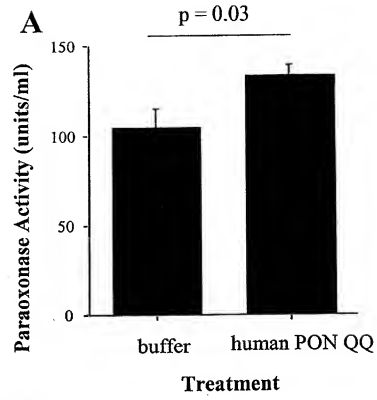

Arylesterase Activity (U/ml)
170
160
150
140
130
120
0
10
20
30
40
50
Time (hours)
Mouse #:
1
2
3

FIGURE 1


A
p = 0.03
150
100
50
0
Paraoxonase Activity (units/ml)
buffer
human PON QQ
Treatment

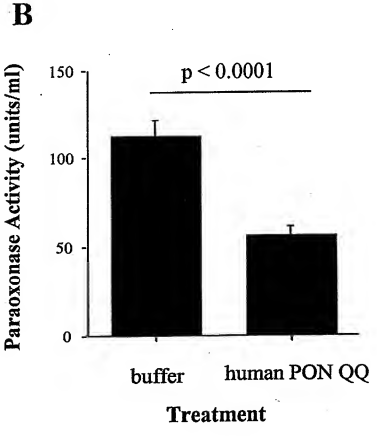

B
p < 0.0001
150
100
50
0
Paraoxonase Activity (units/ml)
buffer
human PON QQ
Treatment

Fig. 2

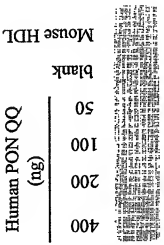


FIGURE 3A

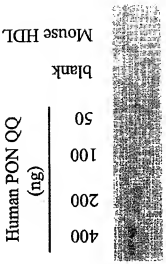


FIGURE 3B

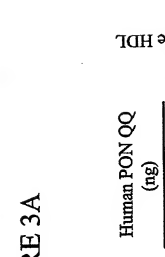


FIGURE 3C

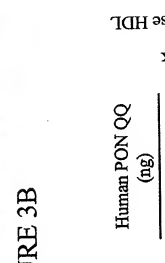


FIGURE 3D

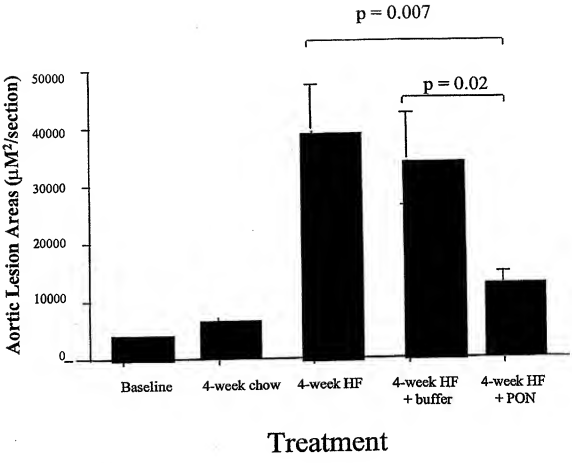

p = 0.007
p = 0.02
Aortic Lesion Areas (μM²/section)
50000
40000
30000
20000
10000
0
Baseline
4-week chow
4-week HF
4-week HF + buffer
4-week HF + PON
Treatment

Fig 4

METHOD OF USING PON-1 TO DECREASE ATHEROMA FORMATION

CROSS-REFERENCE TO RELATED APPLICATION

This application is a division of application Ser. No. 09/199,672, filed Nov. 25, 1998 now U.S. Pat. No. 6,391,298.

BACKGROUND

1. Field of the Invention

The invention relates generally to the field of heart disease and cardiovascular disease. More specifically, the invention is directed to a method of decreasing atheroma formation in mammals, by administration of paraoxonase-1 (PON-1), an expressed protein that has hydrolase activity for organophosphates, and antioxidation activity for low-density lipoprotein (LDL).

2. Background

It is by now well-accepted that atherogenesis and hyperlipidemia are intimately related. Atherogenesis involves the build-up of cholesterol within the endothelium of arterial walls and the subsequent formation of plaques. Plaques can fissure, ultimately causing thrombus formation which may lead to stroke or myocardial infarction. Of the two forms of lipoproteins, high-density (HDL) and low-density lipoprotein (LDL), LDL is positively correlated with plaque formation, while HDL is thought to be anti-atherogenic through the reverse cholesterol transport mechanism (see below).

The lipid transport system is divided into two major pathways, the exogenous pathway (dietary triglycerides and cholesterol absorbed by the intestine) and the endogenous pathway (triglycerides and cholesterol secreted by the liver). The reverse cholesterol transport system, mediated by HDL, is involved in both pathways and is thought to be a major non-receptor based mechanism for removal of cholesterol by HDL. Two subsets of HDL are involved in reverse cholesterol transport, HDL2 and HDL3. Nascent HDL accumulates cholesterol from cell membranes. The circulating enzyme lecithin-cholesterol acyltransferase ("LCAT") associates with HDL and esterifies free cholesterol, causing the esterified cholesterol to move into the core. HDL3 particles accumulate cholesteryl ester, and as it accumulates HDL3 becomes HDL2, which is rich in cholesteryl ester. The cholesteryl ester in HDL2 is then exchanged for triglyceride with the aid of cholesteryl ester transfer protein, converting HDL2 back to HDL3, which is then able to accumulate more free cholesterol. HDL is thought to be antiatherogenic through the reverse cholesterol transport system, because of its ability to take up excess free cholesterol.

Oxidation of LDL is a key intermediate in the formation of atherogenic plaques. It has been found that LDL must undergo modification before it can be ingested by macrophages to form foam cells, which are important components of atherosclerotic plaques (Steinberg, D., et al., "Beyond cholesterol: modifications of low-density cholesterol that increase its atherogenicity," *N. Engl. J. Med.* 320:915 (1989)). In vivo, oxidation is probably the most frequent form of LDL modification. Oxidized LDL not only contributes to the formation of foam cells, but also is chemotactic for circulating monocytes, is cytotoxic, and impairs endothelial function.

HDL was found to inhibit LDL oxidation, which is another potential mechanism by which HDL may reduce

atherosclerosis. S. Parthasarathy and coworkers have shown that incubation of HDL with oxidatively-modified LDL results in inhibition of production of thiobarbituric acid-reactive products (TBARS) (Parthasarathy, S. et al., "High-density lipoprotein inhibits the oxidative modification of low-density lipoprotein," *Biochim. Biophys. Acta* 1044:275 (1990)). However, the mechanism for HDL's antioxidative function remains unknown. In another study, Klimov et al. injected 200 mg of human HDL_2 into rabbits which had been rendered hypercholesterolemic by cholesterol feeding. Total plasma conjugated dienes and trienes were reduced by 20–30% six hours after the injection and remained at that reduced level up to twenty-four hours after the injection (Klimov, A. N., et al., "Antioxidative activity of high density lipoproteins in vivo," *Atherosclerosis* 100:13 (1993)).

Antioxidant therapy has been shown to improve endothelial cell function in patients with hypercholesterolemia and coronary artery disease (Anderson, T J, et al., "The effect of cholesterol-lowering and antioxidant therapy on endothelium-dependent coronary vasomotion," *N. Engl. J. Med.* 332:488 (1995)). The Cambridge Heart Antioxidant Study (CHAOS) randomized 2,002 patients with proven coronary disease to vitamin E, 400 to 800 I.U., or placebo. After a median follow-up of 1.4 years, antioxidant treatment reduced the primary endpoint of cardiovascular death and nonfatal MI by 47 percent (41 v. 64 events) (Stephens, N. G., et al., "Randomized controlled trial of Vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study (CHAOS)," *Lancet* 347:781 (1996)).

Paraoxonase (PON) is a protein secreted by the liver that is found primarily in serum. The name is derived from its ability to hydrolyze the organophosphate paraoxon in vivo. There are 3 known allelic forms of PON. Serum paraoxonase/arylesterase (PON-1) is a 354 residue 43–45 kDa A-esterase associated with HDL (Kelso, G. J., et al., "Apolipoprotein J is associated with paraoxonase in human plasma," *Biochemistry* 33: 832–839 (1994)). It is well-known to be involved in the hydrolysis of several organophosphate insecticides (Murphy, S. D. in *Toxicology: The Basic Science of Poisons*, (eds. Doull, J., Klassen, C., & Amndur, M.) 357–408, Macmillan, New York, (1980); Tafuri, J., et al., "Organophosphate poisoning," *Ann. Emerg. Med.* 16:193–202 (1987)). PON2 and PON3 are known allelic variants that have similar sequences. It is not known if PON2 or PON3 are expressed in vivo. U.S. Pat. Nos. 5,792,639 and 5,629,193 (Human Genome Sciences) are directed to a human paraoxonase gene, its associated vectors and transformed host cells and their use to detoxify organophosphates in vivo and for a neuroprotective effect. The DNA sequences claimed by HGS is likely that of PON2 based on homology searching. An alignment of the PON1 and PON2 nucleic acid sequences shows 69% identity. There is no suggestion in either the '639 or the '193 patents for the use of paraoxonase to reduce atheroma formation described herein.

The physiologic activity of the PON family members was, until recently, unknown. It has recently been postulated that PON may play a role as an in vivo antioxidant that may reduce the peroxidation of LDL (Mackness, M. I., et al., "HDL, its enzymes and its potential to influence lipid peroxidation," *Atherosclerosis* 115:243–253 (1995)). However, the same review stated that other enzymes resident on HDL may also play the same role, such as platelet activating factor acetylhydrolase (Stafforini, D. M., et al., "The plasma PAF acetylhydrolase prevents oxidative modification of low density lipoprotein," *J. Lipid Mediators Cell Signaling* 10:53 (1994)).

Several human population studies have revealed significant associations between the common polymorphisms of the PON1 gene and coronary artery disease (CAD) (Ruiz, J., et al., "Gln-Arg192 polymorphism of paraoxonase and coronary heart disease in type 2 diabetes," *Lancet* 346: 869–872 (1995); Serrato, M., et al., "A variant of human paraoxonase/arylesterase (HUMPONA) gene is a risk factor for coronary artery disease," *J. Clin. Invest.* 96: 3005–3008 (1995)). Also, PON1 has the capacity to destroy certain proinflammatory oxidized phospholipids found in oxidized LDL (Mackness, M. I., et al., "Paraoxonase prevents accumulation of lipoperoxides in low-density lipoprotein," *FEBS Lett* 286: 152–154 (1991); Watson, A. D., et al., "Protective effect of HDL associated paraoxonase-inhibition of the biological activity of minimally oxidized low density lipoprotein," *J. Clin. Invest.* 96: 2882–2891 (1995)). Again, there has been no isolation of the mechanism except to suggest that paraoxonase may be involved.

Mackness et al. ("Is Paraoxonase related to Atherosclerosis," *Chem.-Biol. Interactions* 87:161–171 (1993)) discuss the evidence for an anti-oxidative role for paraoxonase. In this paper they investigated the serum paraoxonase activity in two populations prone to developing atherosclerosis, patients having familial hypercholesterolemia (FH) and IDDM (insulin-dependent diabetes mellitus). They showed a statistically significant increase in the percentage of the population in the low paraoxonase activity group in both FH and IDDM, two diseases manifesting a high occurrence of atherosclerosis. In addition, Mackness et al. studied, in an in vitro LDL oxidation model, the possible role of paraoxonase by adding small amounts in the presence of LDL under oxidative conditions. They concluded that paraoxonase is 300 times more active in preventing LDL oxidation than is HDL or its subfractions. However, they conclude that how paraoxonase protects LDL against oxidation in this model has yet to be determined, and several possibilities are discussed.

In genetic studies with mice, PON1 mRNA and protein levels correlate inversely with aortic lesion size (Shih, D. M., et al., "Genetic-dietary regulation of serum paraoxonase expression and its role in atherogenesis in a mouse model," *J. Clin. Invest.* 97: 1630–1639 (1996)). These data suggest that PON1 activity may bear some relationship to HDL levels and CAD observed in population studies (Tall, A., "Plasma high density lipoproteins: Metabolism and relationship to atherogenesis," *J. Clin. Invest.* 86: 379–384 (1990)).

Familial hypercholesterolemia is a genetic disorder that results in chronically high levels of serum cholesterol, including both HDL and LDL. The disorder is also characterized as an LDL receptor defect. It is autosomal dominant with prevalence estimates of 1 homozygote per million of population. The LDL receptor normally participates in the uptake and subsequent elimination of LDL by hepatocytes. Accumulation of LDL in these patients is a result of the LDL receptor defect. There are definite high-incidence populations due to a founder effect, with French Canadians being the best known. The heterozygotes develop xanthomas at 20–30 years with atherosclerotic heart disease by 40–50 years in males and 50–60 years in females. Homozygotes usually do not survive beyond their thirties, due to cardiac infarctions caused by excessive placque accumulation. They have total cholesterol in the 500–1,000 mg/dl range, develop xanthomas by age 6, and develop symptomatic coronary artery disease by age 10.

Treatment of LDL receptor deficient patients is problematic. Homozygotes do not respond to HMG-CoA reductase inhibitors (they have no functional LDL receptors to upregulate), and heterozygotes respond half as well as normals. Niacin is effective in lowering LDL, but is poorly tolerated. Non-pharmacologic treatment includes weekly plasmapheresis, partial ileal bypass, protocaval shunts, and liver transplants.

There is a clear need for alternative treatments for those patients exhibiting hypercholesterolemia, particularly familial hypercholesterolemia.

SUMMARY OF THE INVENTION

This invention is directed to a method of decreasing atheroma formation in a mammal comprising administering a pharmaceutically effective amount of PON-1 or its functional equivalent. It is shown herein in an animal model that, surprisingly, PON-1 can act to reduce the area of aortic lesions, which are predictive of future atherosclerotic placques (atheromas). This discovery represents a potential pharmacological treatment for FH that should be beneficial for hypercholesterolemia generally.

It is an object of the invention to provide a method for decreasing atheroma formation in a mammal by administering a pharmaceutically effective amount of PON-1 or its functional equivalent, thereby decreasing the potential for atherosclerosis.

It is another object of the present invention to provide a new treatment for those afflicted with FH.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 is a graphical representation of plasma PON-1 (arylesterase assay) activity of LDLR KO mice after receiving an intramuscular (i.m.) injection with 290 units of human PON-1 192Q. Mice were bled at the indicated times (0, 8, 24 and 48 hrs) and PON-1 activities measured.

FIGS. 2A and 2B are bar graphs showing the amount of human PON-1 present in mouse serum 24 hours after injection, either on day 7 (FIG. 2A) or on day 28 (FIG. 2B). Control mice received buffer.

FIGS. 3A through D are Western Blots showing the generation of anti human PON-1 antibodies in mice treated with human PON-1, over the course of the study.

FIG. 4 is a bar graph comparing aortic lesion size in LDLR KO mice in PON-1 treated v. control-treated groups.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

The term "paraoxonase" refers to any of the three known alleles of the glycoprotein enzyme known as paraoxonase ("PON"), namely PON1 PON2 and PON3, and their naturally-occurring variants. PON isolated from serum is called "serum paraoxonase," also PON-1. PON-1 is the only known allele to be expressed, and is present chiefly in serum. In humans there is a known PON-1 variant at amino acid position 192 called the "192Q" variant that has higher anti-atherogenic activity than do the other variants. In populations of European ancestry, the distribution seems to be polymorphic, with low and high activity sub-forms. 192R is the low-activity variant. The term "PON-1 and its functional equivalents" means any paraoxonase or fragment, deletion variant, substitution variant or derivative having antioxidative activity towards atherogenic lipids at least as effective as native PON-1.

PON-1, as in other proteins, can be varied at specific amino acid residue positions to create other variants of native PON-1 ("muteins"). These variants may have more,

less or the same native activity, depending on whether the amino acid substitutions affect the active site, the substrate specificity, the folding of the protein, etc. We prefer conservative modifications and substitutions at other positions of PON-1 (i.e., those that have a minimal effect on the secondary or tertiary structure of the protein). Such conservative substitutions include those described by Dayhoff in *The Atlas of Protein Sequence and Structure* 5 (1978), and by Argos in EMBO J., 8:779–785 (1989). For example, amino acids belonging to one of the following groups represent conservative changes:

ala, pro, gly, gln, asn, ser, thr;

cys, ser, tyr, thr;

val, ile, leu, met, ala, phe;

lys, arg, his;

phe, tyr, trp, his; and

asp, glu.

We also prefer modifications or substitutions that do not introduce sites for additional intermolecular crosslinking or incorrect disulfide bond formation. For example, PON-1 is known to have 2 cysteine residues, at wild-type positions 41 and 352 of the mature sequence.

PON-1 can be isolated from human serum or human plasma (Gan, K. N., et al., "Purification of human serum paraoxonase/arylesterase. Evidence for one esterase catalyzing both activities," Drug Metab. Dispos. 19(1):100–6 (1991)) or can be made through recombinant methods. U.S. Pat. Nos. 5,792,639 and 5,629,193 are directed to PON2 genes, proteins and methods of making and using PON-2, and those expression methods are expressly incorporated herein in their entirety. One of ordinary skill is able to use the teachings therein, and combine them with the known sequence of PON1 (SwissProt Accession No. Q16052; ID PON1 HUMAN) to express the PON-1 protein without undue experimentation.

Similarly, if one wished to produce the 192Q or any other variant, a DNA sequence is constructed by isolating or synthesizing a DNA sequence encoding the wild type PON and then changing the native codon for position 192 to a desired codon by site-specific mutagenesis. This technique is well known. See, e.g., Mark et al., "Site-specific Mutagenesis Of The Human Fibroblast Interferon Gene", *Proc. Natl. Acad. Sci. USA* 81, pp. 5662–66 (1984); and U.S. Pat. No. 4,588,585, incorporated herein by reference.

The biological activity of the PON-1 glycoprotein of this invention can be assayed by any suitable method known in the art. Two methods are disclosed herein, the paraoxonase assay, and the arylesterase assay. (See I. Methods, below).

Pharmaceutical Compositions

The PON-1 protein (also referred to herein as "active compounds") of the invention can be incorporated into pharmaceutical compositions suitable for administration. Such compositions typically comprise the protein and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifingal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. For instance, it is known that PON-1 is a calcium-dependent protein, having several calcium binding loops (Sorenson et al., "Reconsideration of the catalytic center and mechanism of mammalian paraoxonase/arylesterase," Proc. Natl. Acad. Sci. USA 92: 7187–7191 (1995)). Supplementary active compounds can also be incorporated into the compositions.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, intradermal, subcutaneous, oral (e.g., inhalation), transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid, tocopherol or sodium bisulfite; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, N.J.) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound (e.g., a PON-1 protein or anti-PON1 antibody) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. Stabilizers such as albumin, HDL or a sugar such as sucrose, or calcium ions may also be included to increase the shelf-life of the protein.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds are delivered in the form of an aerosol spray from a pressured container or dispenser which contains a suitable propellant, e.g., a gas such as carbon dioxide, or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

The compounds can also be prepared in the form of suppositories (e.g., with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems.

Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Pat. No. 4,522,811.

It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

General guidance regarding dosage and compositions is available in *Remington's Pharmaceutical Science* by E. W. Martin, hereby in corporated by reference.

The invention is further directed to a method of diagnosing predisposition to hypercholesterolemia by assessing the level of native circulating PON-1 in a mammal. The results of the animal testing presented herein show that PON-1 administration can reduce, by a surprising factor, the build-up of fatty streaks in mouse aortic tissues. Thus, detection and monitoring of PON-1 levels in a mammal may be diagnostic of subsequent atheroma formation, which is predictive of atherosclerosis. Assays such as are presented here for paraoxonase or arylesterase may be the basis of such a diagnostic method. The method may take into account the genetic differences between sub-populations which express phenotypic variations in PON-1 which are correlated with higher than normal cardiovascular events. For instance, the PON-1 192Q phenotype has been correlated with higher paraoxonase activity than the 192R phenotype.

It would be expected that an assay may be based on measuring either phenotype separately, and/or the ratio of these two phenotypes present in an individual in order to predict their individual susceptibility to atherosclerosis.

Also contemplated is the use of DNA sequences encoding PON-1 in gene therapy applications. Gene therapy applications contemplated include treatment of those diseases in which PON-1 is expected to provide an effective therapy due to its ability to decrease lipid oxidation such as atherosclerosis, and diseases that are otherwise responsive to lipid oxidation levels. Familial hypercholesterolemia, which is manifested by the lack of expression of LDL receptors, is one such disease that results in very high levels of circulating cholesterol, triglycerides and related lipids.

Local delivery of PON-1 using gene therapy may provide the therapeutic agent to the target area. Both in vitro and in vivo gene therapy methodologies are contemplated. Several methods for transferring potentially therapeutic genes to defined cell populations are known. See, e.g., Mulligan, "The Basic Science Of Gene Therapy", *Science*, 260: 926–31 (1993). These methods include:

1) Direct gene transfer. See, e.g., Wolff et al., "Direct Gene transfer Into Mouse Muscle In Vivo", *Science*, 247:1465–68 (1990);

2) Liposome-mediated DNA transfer. See, e.g., Caplen at al., "Liposome-mediated CFTR Gene Transfer To The Nasal Epithelium Of Patients With Cystic Fibrosis", *Nature Med.* 3: 39–46 (1995); Crystal, "The Gene As A Drug", *Nature Med.* 1:15–17 (1995); Gao and Huang, "A Novel Cationic Liposome Reagent For Efficient Transfection Of Mammalian Cells", *Biochem. Biophys. Res. Comm.*, 179:280–85 (1991);

3) Retrovirus-mediated DNA transfer. See, e.g., Kay et al., "In Vivo Gene Therapy Of Hemophilia B: Sustained Partial Correction In Factor IX-Deficient Dogs", *Science*, 262:117–19 (1993); Anderson, "Human Gene Therapy", *Science*, 256:808–13 (1992).

4) DNA Virus-mediated DNA transfer. Such DNA viruses include adenoviruses (preferably Ad-2 or Ad-5 based vectors), herpes viruses (preferably herpes simplex virus based vectors), and parvoviruses (preferably "defective" or non-autonomous parvovirus based vectors, more preferably adeno-associated virus based vectors, most preferably AAV-2 based vectors). See, e.g., Ali et al., "The Use Of DNA Viruses As Vectors

For Gene Therapy", *Gene Therapy*, 1:367–84 (1994); U.S. Pat. No. 4,797,368, incorporated herein by reference, and U.S. Pat. No. 5,139,941, incorporated herein by reference.

The choice of a particular vector system for transferring the gene of interest will depend on a variety of factors. One important factor is the nature of the target cell population. Although retroviral vectors have been extensively studied and used in a number of gene therapy applications, these vectors are generally unsuited for infecting non-dividing cells. In addition, retroviruses have the potential for oncogenicity.

Adenoviruses have the advantage that they have a broad host range, can infect quiescent or terminally differentiated cells, such as neurons or hepatocytes, and appear essentially non-oncogenic. See, e.g., Ali et al., supra, p. 367. Adenoviruses do not appear to integrate into the host genome. Because they exist extrachromosomally, the risk of insertional mutagenesis is greatly reduced. Ali et al., supra, p. 373.

Adeno-associated viruses exhibit similar advantages as adenoviral-based vectors. However, AAVs exhibit site-specific integration on human chromosome 19. Ali et al., supra, p. 377.

In a preferred embodiment, the PON-1 encoding DNA of this invention is used in gene therapy for lipid-based disorders such as FH, and cardiovascular complications arising from other disorders such as non-insulin dependent diabetes mellitus.

According to this embodiment, gene therapy with DNA encoding PON-1 or muteins of this invention is provided to a patient in need thereof, concurrent with, or immediately after diagnosis.

The skilled artisan will appreciate that any suitable gene therapy vector containing PON-1 DNA or DNA of muteins of PON-1 may be used in accordance with this embodiment. The techniques for constructing such a vector are known. See, e.g., Anderson, W. F., "Human Gene Therapy," *Nature*, 392 25–30 (1998); Verma, I. M., and Somia, N., "Gene Therapy-Promises, Problems, and Prospects," *Nature*, 389 239–242 (1998). Introduction of the PON-1 DNA-containing vector to the target site may be accomplished using known techniques.

This invention is further illustrated by the following examples, which should not be construed to limit the invention, but serve to support it. The content of all patents, patent applications and references referred to herein are hereby incorporated in their entirety.

EXAMPLES

I. Methods

Mice and Diet. The LDLR KO mouse model is a recognized animal model for predicting pharmacological activity for pharmaceutical candidates in the field of atherogenesis. Their LDL receptors, necessary for removal of atherogenic LDL, have been deleted by homologous recombination (Ishibashi S., et al., "Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery," J. Clin. Invest. 92(2) :883–893 (1993)). These mice are particularly susceptible to atheroma formation (fatty streaks) in their primary arteries if fed a high-fat diet. Two month-old female LDL receptor knockout (LDLR KO) mice were purchased from Jackson Laboratory (Bar Harbor, Me.) and maintained on a 6% fat chow diet (Harlan Teklad, Madison, Wis.). For the PON replacement study, 50 female LDLR KO mice, at 3 months of age, were divided into the following 5 groups with 10 mice/group:

Group 1: baseline group, sacrificed on day 0.

Group 2: 4-week chow group, these mice were fed the 6% fat chow diet and sacrificed on day 28. PON-1 activities were measured by paraoxonase assay in groups 4 and 5.

Group 3: 4-week high fat diet group, these mice were fed the high fat diet for 4 weeks and sacrificed on day 28.

Group 4: 4-week high fat diet plus buffer injection, these mice were fed the high fat diet for 4 weeks (day 1 to day 28) and received 80 μl of buffer injections via i.m. on day 1, 3, 6, 8, 10, 13, 15, and 17. The mice then received 80 μl of buffer injections via i.p. on day 20, 21, 22, 23, 24, 25, 26, 27. The mice were then sacrificed on day 28.

Group 5: 4-week high fat diet plus PON injection, these mice were fed the high-fat diet for 4 weeks (day 1 to day 28) and received 80 μl (800 μg, 290 units of paraoxonase activity) of human PON-1 192Q (PON-1 was purified from human plasma donors homozygous for the 192Q mutation, using the method described by Bert L.A Du (Gan, K. N., et al., "Purification of human serum paraoxonase/arylesterase. Evidence for one esterase catalyzing both activities," *Drug Metab. Dispos.* 19(1): 100–106 (1991)) injections via i.m. on day 1, 3, 6, 8, 10, 13, 15, and 17. The mice then received 80 μl of human PON-1 192Q injections via i.p. on day 20, 21, 22, 23, 24, 25, 26, 27. The mice were then sacrificed on day 28.

The high-fat diet contained 15.75% fat, 1.25% cholesterol and 0.5% sodium cholate (Teklad, Madison, Wis.). For groups 4 and 5, plasma PON1 activities were measured on both day 7 (24 hours after an i.m. injection) and day 28 at sacrifice (24 hours after an i.p. injection). For groups 1, 2, and 3, PON-1 activities were measured in plasma samples collected at sacrifice. At sacrifice, the mice were fasted overnight and killed. Blood, hearts, and livers were collected for further analysis.

PON-1 activities and lipid assays. PON-1 activities were measured either by paraoxonase assay using the organophosphate paraoxon as the substrate (Furlong, C. E. et al., "Spectrophotometric assays for the enzymatic hydrolysis of the active metabolites of chlorpyrifos and parathion by plasma paraoxonase/arylesterase," *Anal. Biochem.* 180: 242–247 (1989)) or by arylesterase assay using phenyl acetate as the substrate (Furlong, C. E., et al., "Role of genetic polymorphism of human plasma paraoxonase/arylesterase in hydrolysis of the insecticide metabolites chlorpyrifos oxon and paraoxon," *Am. J Hum. Genet.* 43:230–238 (1988)). Briefly, for the paraoxonase assay, 5 μl of plasma was mixed with the substrate solution containing 1.2 mM of paraoxon, 2.0 M NaCl, 0.1 M Tris HCl pH 8.5 and 2.0 mM $CaCl_2$. The production of p-nitrophenol was measured, at room temperature, as change in absorbance (O.D.) at 405 nm over 5 minutes. A standard curve was also constructed by measuring O.D.$_{405}$ of various concentrations of p-nitrophenol. One unit of paraoxonase activity is defined as 1 nmole of p-nitrophenol produced/min. For arylesterase assay, 1 μl of plasma was mixed with 1 ml of substrate solution containing 3.26 mM phenylacetate, 9.0 mM Tris HCl pH 8.0, and 0.9 mM $CaCl_2$. The production of phenol was measured, at room temperature, as change in absorbance (O.D.) at 270 nm over 2 minutes. The arylesterase activity was then calculated using the molar extinction coefficient for phenol (1,310 M^{-1} cm^{-1}). One unit of arylesterase activity is defined as 1 μmole phenol produced/min. For all of the groups, plasma total cholesterol, HDL cholesterol, VLDL/LDL cholesterol, and triglycerides were measured in

plasma samples collected at sacrifice using enzymatic procedures employing enzymatic end points (Mehrabian, M., et al., "Influence of the apoA-II gene locus on HDL levels and fatty streak development in mice," *Arterioscler. Thromb.* 13: 1–10 (1993)).

Aortic lesion measurements. Briefly, the use of aortic lesion measurements requires that, at sacrifice, the upper portion of the heart and proximal aorta is obtained and embedded in OCT compound (Tissue-Tek OCT Compound—Sakura Finetek USA Inc., Torrence, Calif.) and frozen. Every other 10-μm thick cryosection, beginning where the aortic valves appear, is collected for a distance of about 500 μm. These sections are stained with oil red O (Oil Red O, Sigma Chemical Company, St. Louis, Mo.) and counter-stained with hematoxylin (Hematoxylin aqueous formula, Biomeda Corp., Foster City, Calif.) and Fast Green (Fast Green FCF, Sigma, St. Louis, Mo.). The lipid containing areas on 25 sections are determined using a microscope eyepiece grid. Mean lesion area/section are then calculated (Mehrabian, M., et al., "Influence of the apoA-II gene locus on HDL levels and fatty streak development in mice," *Arterioscler. Thromb.* 13: 1–10 (1993)).

Western blot analysis. Various amounts of purified human PON-1 192Q (400, 200, 100, 50 ng/lane) and 1 μl of mouse HDL (equivalent to 6 μl of mouse plasma) were loaded on denaturing polyacrylamide gel for electrophoresis (denaturing agent used: 2×buffer: 0.5 M Tris-HCl, pH6.8, 2.5 ml; glycerol, 2 ml; 10% SDS, 4 ml; 0.1% bromophenol blue, 0.5 ml; beta-mercaptoethanol, 0.5 ml; water to 10.0 ml). The fractionated proteins were then transferred onto nitrocellulose paper (Hybond-ECL nitrocellulose, Amersham, Buckinghamshire, UK). Four of such identical nitrocellulose blots were than incubated for 1 hour with one of the following solutions: (1) 1:500 dilution of pooled plasma from mice injected with buffer after 4 weeks of injection, (2) 1:500 dilution of pooled plasma from mice injected with human PON-1 192Q after 4 weeks of injection, (3) 1:500 dilution of pooled plasma from mice injected with human PON-1 192Q after 1 week of injection, (4), 1:1000 dilution of a rabbit antibody against mouse PON-1 (rabbit anti-mouse PON-1: was generated using recombinant mouse PON-1 expressed in *E. coli* at UCLA by Diana Shih and Ling-jie Gu, unpublished results). The blots were then washed with PBS containing 0.1% Tween-20 and then incubated for 1 hour with anti-rabbit IgG secondary antibodies conjugated with HRP (Amersham, Buckinghamshire, UK). The blots were then washed and the image was visualized using the ECL Western blotting detection reagents from Amersham (Amersham, Life Science, Inc., Arlington Heights, Ill.).

II. EXAMPLES

Example 1

Time Course Study of Plasma PON-1 Activity in LDLR KO Mice Injected with Human PON-1 192Q

Three female LDLR KO mice, maintained on chow diet, were each injected i.m. with 290 units of human PON-1 192Q. Blood samples were collected immediately before the injection time 0) and 8, 24, and 48 hours after the injection. Plasma PON-1 activities were then measured using arylesterase assay. As shown in FIG. 1, the mean plasma PON-1 activity at 8, 24, and 48 hr after injection was 107%, 125%, 109% that of time 0 (time 0 vs. 24-hr, p=0.003). Our data indicate that i.m. injection is an effective way to deliver PON-1 into mice. However, the increases in PON-1 activities in these mice were smaller than what we have expected.

Example 2

PON1 192Q Replacement Study

The experimental design was previously described in Methods. For Group 4 (4-week high fat diet plus buffer injection) and Group 5 (4 week high-fat diet plus 290 units human PON-1 192Q injection), plasma PON-1 activities were measured on day 7, 24 hr after the third i.m. injection. At day 7 there were 10 mice in each group; values shown are averages from two independent paraoxonase assays. At day 28, there were 9 mice in each group; paraoxonase assay.

As shown in FIGS. 2A and 2B, we found that, on day 7, the high-fat diet plus PON-1 injection group exhibited 27% higher PON-1 activities as compared to the high fat plus buffer injection group (p=0.03) (FIG. 2A). However, on day 28 (FIG. 2B), we found that the high fat diet plus PON-1 injection group had only 50% of the PON-1 activities as compared to those of the high-fat plus buffer injection group (p<0.0001). We postulated that the reduction of PON-1 activities in the PON-1 injected group on day 28 was likely caused by an immune response in the mice toward the injected human PON-1 192Q.

Example 3

Detection of Anti-human PON-1 Antibodies in LDLR KO Mice Injected with Human PON-1.

Various amounts of purified human PON1 192Q (400, 200, 100, 50 ng/lane) and 1 μl of mouse HDL (equivalent to 6 μl of mouse plasma) were loaded on denaturing polyacrylamide gel for electrophoresis. The fractionated proteins were then transferred onto nitrocellulose paper. Specifically with regard to FIGS. 3A through D, four of these identical nitrocellulose blots were then incubated for 1 hour with one of the following solutions: panel A, 1:500 dilution of pooled plasma from mice injected with buffer after 4 weeks of buffer injection; panel B, 1:500 dilution of pooled plasma from mice injected with human PON-1 192Q after 4 weeks of injection; panel C, 1:500 dilution of pooled plasma from mice injected with human PON-1 192Q after 1 week of injection; and panel D, 1:1000 dilution of a rabbit antibody against mouse PON-1. The blots were then washed with PBS containing 0.1% of Tween-20 and incubated for 1 hour with secondary antibodies conjugated with HRP. The blots were then washed and the image was visualized using the ECL technique. As shown in panel A, the 4-week buffer-injected mice did not have antibodies against human PON-1 in their plasma, while the 4-week PON-1-injected mice contain antibodies against human PON1 in their plasma (panel B). However, these antibodies did not cross react with the mouse PON (Panel B). We did not detect any anti-human PON-1 antibodies in the pooled plasma of 1-week PON-1-injected mice (panel C). As shown in panel D, a rabbit anti-mouse PON-1 antibody detected both the mouse PON-1 and, to a lesser extent, the human PON-1.

It is unlikely that the reduction of PON-1 activities in the PON-1 injected animals on day 28 is directly caused by the interaction between the mouse PON-1 and the antibodies since the antibodies against human PON-1 did not cross react with the mouse PON1 protein. Since the human PON-1 is likely to reside on the same HDL particles as the mouse PON-1, recognition of the PON-1 containing HDL particles by the antibodies may enhance the clearance of these HDL particles, thus causing the removal of mouse PON-1 on the same particles.

Example 4

Lipid Levels.

Lipid levels of plasma samples collected at sacrifice were examined. As shown in Table 1, there were no significant

differences in plasma lipid levels between the 4-week high-fat plus buffer-injected group and the 4-week high fat-group. Interestingly, as compared to the buffer-injected mice, the PON-1-injected mice had a moderate decrease in both total cholesterol (p=0.04) and VLDL/LDL cholesterol (p=0.04) levels, a moderate increase in triglycerides (p=0.005), and no difference in HDL cholesterol level. Therefore, the PON-1-injected mice had a less atherogenic lipid profile as compared to the buffer-injected group.

TABLE 1

Plasma PON-1 activities and lipid levels of LDLR KO mice at sacrifice

	PON-1 activity[1]	Total Cholesterol[2]	VLDL/LDL Cholesterol[2]	HDL Cholesterol[2]	Triglycerides[2]
Baseline	329 ± 21	291 ± 15	206 ± 17	86 ± 3	162 ± 16
4-Week Chow	420 ± 11	332 ± 10	230 ± 10	101 ± 2	318 ± 25
4-Week HF[3]	105 ± 6	2315 ± 175	2289 ± 174	27 ± 3	44 ± 11
4-Week HF[3] + Buffer	112 ± 9	2213 ± 145	2190 ± 145	23 ± 2	26 ± 4
4-Week HF[3] + PON-1	56 ± 5	1862 ± 63	1836 ± 62	26 ± 2	44 ± 4

[1]Values shown are mean ± S.E. of 9 or 10 animals in each group. The units are units of paraoxonase activity/ml plasma.
[2]Values shown are mean ± S.E. of 9 or 10 animals in each group. The units are mg/dL.
[3]HF means high fat diet.

Example 5

Aortic Lesions.

We then examined the aortic fatty streak formation in these mice. Three-month old female LDLR KO mice (10 mice/group) were either sacrificed at time 0 (baseline group), fed the chow diet for 4 weeks (4-week chow), fed the high-fat diet for 4 weeks (4-week HF), fed the high fat diet plus buffer injections (4-week HF+buffer), or fed the high-fat diet plus human PON-1 192Q injections (4-week HF+PON). At the end of 4-week treatments, mice were sacrificed. The hearts were collected and aortic lesion areas were scored. For Group 4 (4-week HF+buffer) and Group 5 (4-week HF+PON), the lesion sizes were scored once in a non-blind and once in a blind fashion to avoid bias. The scores from both times were very similar. We found no significant difference in aortic lesion sizes between the 4-week high-fat and the 4-week high-fat plus buffer injection group (FIG. 4). From the non-blind scoring, the mean lesion sizes of the buffer injected (Group 4) and PON-1-injected (Group 5) groups were 34222±8008 and 12864±1985 mm^2/section, respectively. From the blind scoring, the mean lesion sizes of the buffer injected and PON-1-injected groups were 32422±7470 and 13383±1850 mm^2/section, respectively. Therefore, we obtained very similar results using either blind or non-blind methods, demonstrating that the 4-week high-fat plus PON-1 192Q injection group had significantly less aortic lesion area than both the 4-week high-fat plus buffer-injected group and the 4-week high-fat group (FIG. 4). The results suggest that PON-1 replacement is an effective way to reduce aortic fatty streak lesion in the LDLR KO mice.

In summary, it was found that the PON-1 192Q-injected mice had elevated PON-1 activities on day 7, and lower levels of PON-1 activities on day 28 as compared to the buffer-injected mice. However, when aortic lesion formation was examined on day 28, a surprizingly large 60% (approximate) reduction in aortic lesion size was found in PON-1 192Q-injected mice as compared to the buffer-injected mice. Possible explanations for the low PON-1 activity levels in PON-1-treated mice on day 28 are as follows: First, the generation and the titer of antibodies against human PON-1 192Q in PON-1 injected mice probably started late and remained low until the end of the experiment. Therefore, for most of the time during the study, the injected PON-1 192Q was effective in preventing LDL oxidation and, thus, atherosclerosis. Second, if one assumes that PON-1 is most effective in preventing the initiation of atherosclerosis, that is prevention of LDL oxidation and therefore prevention of inflammatory response and reduced recruitment of monocytes into subendothelial cells (Mackness, M. I., et al, supra; Watson, A. D., et al., supra; Shih, D. M., et al., "Mice lacking serum paraoxonase are susceptible to organophosphate toxicity and atherosclerosis," *Nature* 394:284–287 (1998)), then the PON replacement might have been most effective during the first 2 weeks of the study rather than the last 2 weeks of the study. Therefore, even though PON-1-injected mice had less PON-1 activity than the buffer-injected mice at the end of the 28-day experiment, they still developed less aortic lesions. Those skilled in the art will be able to recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. For instance, PON-2 and PON-3 may have antioxidant activity similar to that of PON-1, and may be equally useful in the method of this invention. Also, other deletion or substitution mutations may provide similar functional equivalents. Such equivalents are intended to be encompassed by the spirit and scope of the following claims.

I claim:

1. A method of decreasing atheroma formation in a mammal comprising administering a pharmaceutically effective amount of recombinant PON-1, polypeptide, wherein reduction in aortic lesion formation is taken as a measure of decreased atheroma formation.

2. The method of claim 1, wherein said effective amount ranges from about 0.1 μg/kg of body weight to about 100 mg/kg.

3. The method of claim 1, wherein said PON-1 comprises recombinant human PON-1.

4. The method of claim 1, wherein said PON-1 comprises PON-1 192Q.

5. A pharmaceutical composition for decreasing atheroma formation, comprising administering recombinant PON-1 protein to a patient in need thereof comprising recombinant

PON-1 and a pharmaceutically acceptable carrier, wherein reduction in aortic lesion formation is taken as a measure of decreased atheroma formation.

6. A method of decreasing atheroma formation in a mammal comprising administering a pharmaceutically effective amount of recombinant PON-1 DNA, or of a mutein of PON-1 DNA having PON-1 equivalent function, to a mammal such that functional PON-1 is detectable in said mammal, wherein reduction in aortic lesion formation is taken as a measure of decreased atheroma formation.

7. A method of decreasing atheroma formation in a mammal comprising administering a pharmaceutically effective amount of a recombinant polypeptide that is recognized by a PON-1-specific antibody, wherein reduction in aortic lesion formation is taken as a measure of decreased atheroma formation.

8. The method of claim 7, wherein said polypeptide has paraoxonase and arylesterase activity and specifically binds apoJ.

9. The method of claim 7, wherein said polypeptide is in a pharmaceutically acceptable carrier.

10. The method of claim 7, wherein said polypeptide is recombinant human PON-1.

* * * * *

EXHIBIT 2

ELSEVIER

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Toxicology letters

Paraoxonase protects against chlorpyrifos toxicity in mice

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Abstract

Paraoxonase can hydrolyze paraoxon (PO), chlorpyrifos-oxon (CPO) and other organophosphates. Previous studies have indicated that the levels of serum paraoxonase can influence the toxicity of PO and CPO. In the present study we have investigated whether exogenous paraoxonase administered to mice would offer protection toward the acute toxicity of a phosphorothioate, chlorpyrifos (CPS). Paraoxonase was purified from rabbit serum and injected i.v., or i.v. plus i.p., in mice. Inhibition of acetylcholinesterase (AChE) in brain, diaphragm, plasma and red blood cells was measured as an index of CPS (100 mg/kg) toxicity. Administration of paraoxonase 30 min before CPS increased plasma enzyme activity toward CPO by 35-fold, and protected against its toxicity; protection was still present at 24 h, when enzyme activity was still 20-fold over basal. When paraoxonase was given 30 min after CPS, a significant protection against CPS toxicity was still observed, while after 3 h the protective effect was decreased. To mimic conditions of severe acute poisoning, a higher dose of CPS (150 mg/kg) was also administered. Administration of paraoxonase 30 min after this exposure abolished cholinergic signs and significantly protected against AChE inhibition. These results indicate that exogenous paraoxonase offers significant protection against CPS toxicity when administered both before and after the organophosphate, suggesting that it may be considered as a potential additional treatment of organophosphate poisoning.

Keywords: Chlorpyrifos; Paraoxonase; Acetylcholinesterase inhibition; Organophospate toxicity; Mice

1. Introduction

Paraoxonase (EC 3.1.1.2) is an esterase first recognized by Aldridge [1] as being able to hydrolyze the organophosphate paraoxon (PO) to *p*-nitrophenol and diethylphosphoric acid. Paraoxonase is present in several mammalian tissues, with liver and blood having the highest activity (reviewed in [2]). In addition to PO, paraoxonase can hydrolyze a number of other organophosphates, such as chlorpyrifos-oxon (CPO), pyrimifos oxon, diazinon oxon, as well as certain aromatic esters such as phenyl acetate [3].

Human serum paraoxonase exhibits a genetic polymorphism [4], which is inherited according to a single Mendelian two-allele trait at a single autosomal locus. Three phenotypes of paraoxonase have been found in population studies: in-

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dividuals homozygous for the low activity allele (A-type) or for the high activity allele (B-type), or heterozygotes (AB-type). The paraoxonase polymorphism has been studied in several populations, and a clear bimodal distribution has been found in Caucasian populations (reviewed in [2]), with a gene frequency of 0.69 for the low activity allele and of 0.31 for the high activity allele [5]. The genetic polymorphism of paraoxonase activity is substrate-dependent. In addition to PO, the polymorphism is also observed with the oxons of methylparathion, chlorthion and EPN (reviewed in [2]), but not with phenyl acetate or CPO [5]. In addition to qualitative genetic polymorphism, a remarkable variation in serum paraoxonase levels within a genetic class is also present. Western blot experiments have indicated that the variation in serum paraoxonase activity corresponds to differences in protein levels [6].

Human serum paraoxonase has been purified to homogeneity [7,8] and evidence suggests that human serum contains a single enzyme with both paraoxonase and arylesterase activity. Purified paraoxonase is a glycoprotein with a minimal molecular weight of about 43 000, with carbohydrate representing about 15.8% of the total weight [8]. Calcium is required for both enzymatic stability and catalytic activity [7]. Paraoxonase is very closely associated with the high-density lipoprotein (HDL) fraction of serum, suggesting a role for this enzyme in lipoprotein metabolism [9].

Rabbit and human paraoxonase cDNAs have been cloned and sequenced [10,11]. The rabbit and human cDNA clones are extensively conserved in nucleotide and deduced amino acid sequences (86% homology). Northern blot hybridization experiments have shown that paraoxonase is synthesized predominantly in liver and Southern blot experiments indicate that it is encoded by a single gene [10]. Two polymorphic sites have been identified in human paraoxonase: Leu/Met at position 55 and Arg/Gln at position 192, and the polymorphism in enzyme activity has been ascribed to the latter [12], with low activity homozygotes (A-type) presenting Gln in position 192. cDNAs for mouse or rat paraoxonase have not been cloned yet.

It has long been suggested that paraoxonase polymorphism may influence human susceptibility to organophosphorus insecticide toxicity [13–15]. Animal studies suggest that this may be the case. The greater toxicity of organophosphates in birds, compared to mammals, correlates with the very low levels of serum paraoxonase activity in avian species [16]. Rabbits, which have a 7-fold higher enzyme activity than rats, have a 4-fold higher tolerance to PO toxicity [17]. Furthermore, administration of exogenous paraoxonase to rats has been shown to offer protection against the toxicity of PO and CPO [18,19].

The present study was undertaken to expand these latter findings in another species, the mouse, in order to provide additional support for the hypothesized significant role played by paraoxonase in influencing susceptibility to organophosphate toxicity. In particular, we addressed the questions of whether exogenous paraoxonase would affect the toxicity of a phosphorothioate, in addition to the oxon, and would be useful as a therapeutic agent when administered after organophosphate poisoning.

2. Materials and methods

2.1. Animals and chemicals

All animals used in this study were female BALB/c mice (aged 8–10 weeks; body weight 17–21 g). Animals were housed 5 per cage and had water and food ad libitum. Chlorpyrifos (CPS, *O,O*-diethyl-*O*-(3,5,6-trichloropyridyl)-phosphorothioate, 98.8% purity) was obtained from Chem Services (West Chester, PA). CPO (*O,O*-diethyl-*O*-(3,5,6-trichloropyridyl)-phosphate, 96.2% purity) was a gift from Dow Chemical Company (Midland, MI). PO (diethyl *p*-nitrophenyl phosphate, > 99% purity) was obtained from ICN Biomedicals Inc. (Plainview, NY).

2.2. Purification of rabbit serum paraoxonase

Paraoxonase/arylesterase was purified from rabbit serum (Northwest Rabbit Company, Port Orchard, WA), as previously described in detail [7].

2.3. Animal treatments

Purified rabbit serum paraoxonase in G-75 elution buffer (50 μl; 3.34 units of paraoxonase, 41.7 units of chlorpyrifos-oxonase) was injected i.v.

(tail vein) and i.p. as noted for each experiment. Control animals received no injections. Blood for enzyme assay was drawn from the postorbital vein of the eye to assure equilibration of the enzyme with the serum. CPO (14 mg/kg) or CPS (100 mg/kg or 150 mg/kg) was dissolved in acetone, and a volume of 20 μl was applied to a previously shaven 4-cm^2 area on the back of the mice. These dosages were chosen on the basis of preliminary experiments. Control animals received the same volume of acetone. After 4 h blood was withdrawn from the postorbital vein, mixed with heparin, and kept on ice. Animals were then terminated by cervical dislocation, and brain and diaphragm were rapidly removed on ice.

2.4. Assay of paraoxonase and chlorpyrifos-oxonase activities

Paraoxonase and chlorpyrifos-oxonase activities were determined in plasma by methods developed by Furlong et al. [5,20]. The stock assay buffer contained 2.0 M NaCl, 0.1 M Tris–HCl, pH 8.5, and 2.0 mM $CaCl_2$. For the paraoxonase assay, 1.2 mM PO in assay buffer was made fresh, and 10–15 min of vigorous stirring was required to assure maximum and complete solubility. For each reaction, 1–10 μl plasma was added to 1 ml PO assay solution and initiated at 37°C. The absorbance of *p*-nitrophenol was continuously monitored for 4 min at 405 nm. For the chlorpyrifos-oxonase assay, a 30-mM solution of CPO in 100% methanol was prepared. The incubation mixture contained 1–10 μl plasma and 1 ml stocking assay buffer. The reaction was initiated at 37°C by adding 10 μl CPO solution to the mixture, and the absorbance of 3,5,6-trichloro-2-pyridinol was continuously monitored in 2 min at 310 nm. Enzyme activity is expressed as units/l (unit = μmol PO or CPO hydrolyzed/min). Basal levels of paraoxonase in plasma from control (untreated) mice were 238 ± 49 units/l and 3086 ± 692 units/l, with PO or CPO as substrate, respectively (n = 18–20).

2.5. Acetylcholinesterase (AChE) assay

Activity of AChE in brain and diaphragm was assayed according to the method of Ellman et al. [21] as modified by Benke et al. [22]. Plasma and red blood cell (RBC) cholinesterase activities were determined based on the method of Voss and Sachsse [23]. Fresh heparinized blood samples were used in this assay. AChE activity is expressed as μmol ATC hydrolyzed/min/g of wet tissues or ml of blood. In control (untreated) mice, AChE activities were: 16.40 ± 0.64 (brain), 3.08 ± 0.09 (diaphragm), 2.90 ± 0.34 (plasma) and 0.53 ± 0.06 (RBC) (n = 20).

2.6. Data analysis

Student's t-test (unpaired, two-tail) was applied to the means of the control and treatment groups to determine the significance ($P < 0.05$) of any differences in AChE activity [24]. Calculations and analyses were carried out by using Statview statistical software.

3. Results

Previous studies had examined the half-life of rabbit paraoxonase (3.34 units paraoxonase; 41.7 units chlorpyrifos-oxonase) injected into mice by different routes (i.v., i.p. and i.m.; [25]). Based on the results of those studies, 2 types of administration were chosen for the present experiments: i.v. injection, which elevated mouse serum enzyme activity toward CPO by 35-fold at 30 min with a half-life of 6 h; and a combination of i.v. plus i.p. injections which still increased enzyme activity toward CPO by 35-fold, but also increased the half-life to 30 h [25].

To examine whether the increased serum paraoxonase levels would offer protection against cholinesterase inhibition caused by CPO, the chemical was applied dermally, since this is the primary route of exposure to organophosphorus insecticides. In each experiment, 3 groups of animals were used: a control group, which received acetone only; a CPO group, which received CPO dissolved in acetone; and a CPO + CPOase group, which was given paraoxonase via i.v. injection, followed after 30 min by CPO. Serum paraoxonase activity was measured 30 min following enzyme administration, and in all experiments, enzyme activity toward chlorpyrifos-oxon (CPOase) was increased by 25- to 35-fold. Animals were sacrificed 4 h after exposure to CPO. AChE activity in brain, diaphragm, plasma, and RBC was measured as an

index of CPO toxicity. At the dose of 14 mg/kg, CPO caused significant AChE inhibition in various tissues, without causing any significant sign of intoxication. AChE activity was significantly less inhibited in mice pretreated with purified rabbit paraoxonase than in control animals, indicating that i.v. injection of the enzyme provides protection from a dermal CPO challenge. This protective effect was most pronounced in brain and diaphragm, where AChE activity (as % of control) was 12 ± 1 and 23 ± 2 vs. 89 ± 3 and 89 ± 7, respectively ($n = 3–6$; $P < 0.05$). The effect of administration of exogenous paraoxonase on the toxicity of parent compound, CPS, was also examined. The experimental design was identical to that utilized for CPO. The dose of CPS was 100 mg/kg, which caused 50–60% inhibition of AChE activity in brain without any significant cholinergic sign. Intravenously injected paraoxonase afforded protection against CPS toxicity in most tissues, particularly in brain. In this tissue, AChE activity (as % of control) was 41 ± 4 vs. 92 ± 9 ($n = 5$; $P < 0.05$). These results confirm our previous observations [25].

Since the half-life of rabbit paraoxonase given by the i.v. plus i.p. route was very long [25], in an additional experiment mice were challenged with CPS 24 h, instead of 30 min, after the i.v. plus i.p. injections of the enzyme. The dose of CPS (100 mg/kg) was the same as that utilized in the previous experiment, but mice were injected with 6.68

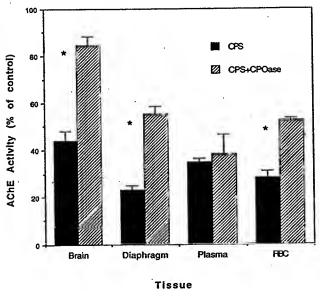

Fig. 1. Inhibition of AChE activity following dermal exposure to CPS (100 mg/kg) in animals which were administered CPS alone (solid bars; CPS; $n = 4–6$) and in animals which were preinjected with 6.68 units of paraoxonase i.v. plus i.p. 24 h prior to challenge (hatched bars; CPS + CPOase; $n = 5–7$). *Significantly different from CPS alone, $P < 0.05$. All results are expressed as percentage of control animals (acetone-treated) and represent the means ± S.E.M.

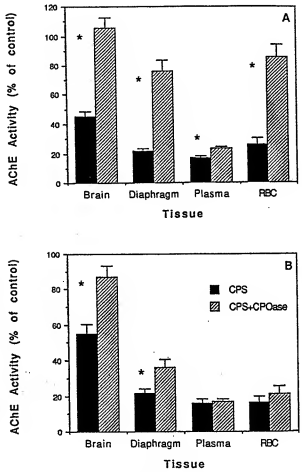

Fig. 2. Inhibition of AChE activity in the indicated tissues following dermal exposure to CPS (100 mg/kg) in animals which were administered CPS alone (solid bars; CPS; $n = 4–6$) and in animals which were injected i.v. with 3.34 units of paraoxonase 30 min (A) or 24 h (B) after challenge (hatched bars; CPS + CPOase; $n = 3–5$). Results are expressed as percentage of control animals (acetone-treated) and represent the means ± S.E.M. *Significantly different from CPS alone, $P < 0.05$.

units of paraoxonase. CPOase activity at 24 h was still 20-fold higher than basal (not shown). As shown in Fig. 1, AChE activity in brain and diaphragm was significantly less inhibited in mice which were treated with paraoxonase 1 day before CPS challenge.

The experiments described above indicate that paraoxonase decreases CPS inhibition of AChE, when injected either 30 min or 24 h before exposure to the chemicals. Additional experiments were carried out to determine whether paraoxonase could still be effective when administered after CPS exposure. For this purpose, mice were given CPS (100 mg/kg) followed by i.v. injection of paraoxonase at different intervals. Cholinesterase activity in various tissues was measured 4 h after CPS administration. When mice received paraoxonase 30 min following CPS exposure, the enzyme did prevent the reduction of cholinesterase activity in all tissues (Fig. 2A). When paraoxonase was injected 3 h after CPS, however, there was only a protective effect in brain and diaphragm, and no effect in plasma and RBC (Fig. 2B).

The dose of CPS (100 mg/kg) used in the above experiments caused significant reductions of cholinesterase activity, but no obvious signs of cholinergic intoxication. Therefore, a higher dose of CPS (150 mg/kg) was selected in the next experiment to test the effectiveness of paraoxonase under conditions of severe acute CPS poisoning. At this dose, acute symptoms developed 2–3 h after CPS poisoning, including weakness, lacrimation, ataxia, and difficulties in breathing. No signs of CPS intoxication were present in mice which received i.v. paraoxonase 30 min following CPS exposure. A striking protection against cholinesterase inhibition was observed in brain and diaphragm (Fig. 3).

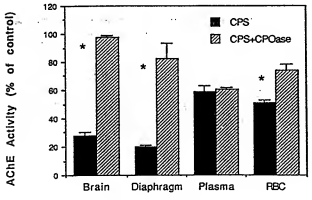

Fig. 3. Inhibition of AChE activity in the indicated tissues following dermal exposure to CPS (150 mg/kg) in animals which were administered CPS alone (solid bars; CPS; $n = 3$) and in animals which were injected i.v. with 3.34 units of paraoxonase 30 min after challenge (hatched bars; CPS + CPOase, $n = 3$). Results are expressed as percentage of control animals (acetone-treated) and represent the means ± S.E.M. *Significantly different from CPS alone, $P < 0.05$.

4. Discussion

The results of these studies indicate that purified rabbit paraoxonase injected into mice increases the serum activity and protects mice from CPS toxicity. Paraoxonase was able to prevent the decrease in cholinesterase activity when it was administered before, as well as after, CPS poisoning.

There has been substantial speculation about the in vivo role of serum paraoxonase in detoxifying organophosphorus insecticides, however, only a few studies with rats have directly approached this question [8,19]. Here, we have expanded our initial observations in mice [25]. The advantages of a mouse model are that much less purified paraoxonase is required for injection than with rats, and that the species is ideal for gene transfer studies, such as the development of transgenic mice with altered paraoxonase levels. The results reported here show that the mouse is suitable for these studies.

The parent thioate compounds, such as parathion and CPS, require conversion to oxons to become active inhibitors to cholinesterase. The thioate compound itself is neither toxic nor a substrate for serum paraoxonase. Previous studies demonstrated that serum paraoxonase provided protection against PO and CPO in rats [18,19], and in mice [25]. Our results expand an initial observation [25], and show that injected rabbit paraoxonase provides excellent protection also against the toxicity of the parent compound CPS.

The other distinctive finding of this study is that high serum paraoxonase was able to prevent the reduction of cholinesterase activity when it was administered after CPS exposure. This finding sug-

gests that the use of paraoxonase in organophosphate poisoning (possibly in combination with other conventional treatments) should be further investigated.

It had been hypothesized that individuals with low serum paraoxonase activity would have a diminished ability to metabolize the oxygen metabolites of organophosphates, and therefore might be more sensitive to the toxicity of certain organophosphates [14,15]. Our finding that levels of serum paraoxonase activity could affect the toxicity of CPS, the parent compound applied as an insecticide, suggests that epidemiological studies may be conducted to test the hypothesis that genetic variations in serum paraoxonase activity may represent a factor in determining susceptibility to poisoning by organophosphorus insecticides.

Raising the serum paraoxonase activity provides very effective protection of cholinesterase inhibition from CPO and CPS toxicity in brain and diaphragm. Particularly in brain, nearly complete protection was found if paraoxonase was injected 30 min after CPS administration. On the other hand, the protective effects toward plasma and RBC cholinesterase varied. The only significant protection in both plasma and RBC was observed when paraoxonase was given 30 min after CPS. In a recent study, it was observed that another organophosphate-hydrolyzing enzyme, phosphotriesterase, could not prevent the decrease of serum cholinesterase activity caused by PO [26]. It has been suggested that the high affinity of organophosphates to the serum cholinesterase might explain this finding. It should be noted, however, that the toxicity of organophosphates is primarily due to the accumulation of free acetylcholine in brain and diaphragm, and, therefore, the protection of cholinesterase inhibition found in these two tissues might be more important than in blood. Indeed, in the experiment with a high dose of CPS (150 mg/kg), paraoxonase alleviated the signs of cholinergic intoxication.

The traditional therapy for organophosphate poisoning consists of a combination of drugs such as atropine and oxime given after exposure. Recently, another suggested approach in the treatment of organophosphate intoxication has been the use of exogenous enzymes as pre-treatment drugs [27]. Compared to the combination of antimuscarinics and reactivators, the pre-treatment with exogenous enzymes may alleviate certain side effects, such as tremors and convulsions. Different kinds of exogenous enzymes have been examined for their effectiveness against organophosphate toxicity. Cholinesterase has been shown to be capable to sequester organophosphates and reduce their toxicity [28]. Organophosphate-hydrolyzing enzymes, like paraoxonase or phosphotriesterase, which are able to degrade a wide variety of organophosphates, have also protective effects [19,26,29; this study].

It may be expected that organophosphate-hydrolyzing enzymes would be much more effective in protecting from organophosphate toxicity. The protection of cholinesterase against organophosphates is via the formation of 1:1 stoichiometric covalent conjugates with the toxic agent. The exogenous cholinesterase is inactivated when it binds to the organophosphate compound, and thus a large amount of cholinesterase will be needed to obtain the significant protection. In contrast to cholinesterase, both paraoxonase and phosphotriesterase are able to catalytically detoxify their organophosphorus substrates over a short time period using small quantities of enzyme. As a treatment for organophosphate poisoning, serum paraoxonase has some advantages compared with other enzymes. The purification procedure of serum paraoxonase has been developed, and pure paraoxonase can be readily prepared in sufficient quantities. Serum paraoxonase is also very stable during storage in the presence of calcium ion. Since the rabbit and human paraoxonase cDNAs have been cloned and sequenced, it should be possible to develop a recombinant system to produce paraoxonase in an economical way. According to our results, the half-life of exogenous paraoxonase in mouse circulation is very long, more than 30 h when administered by i.v. plus i.p. injection, and more than 50 h when given by i.v. plus i.m. injection [25]. Since serum paraoxonase is able to hydrolyze a wide variety of organophosphates, including insecticides, and the nerve agents soman and sarin, with a relatively high turnover number,

this enzyme should be considered as a potential additional treatment for organophosphate poisoning.

Acknowledgements

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References

[1] Aldridge, W.N. (1953) Serum esterase II: an enzyme hydrolysing diethyl *p*-nitrophenyl phosphate (E600) and its identity with the A-esterase of mammalian sera. Biochem. J. 53, 117–124.

[2] Geldmacher-Von Mallinckrodt, M. and Diepgen, T.L. (1988) The human serum paraoxonase — polymorphism and specificity. Toxicol. Environ. Chem. 18, 79–196.

[3] Mackness, M.I., Arrol, S. and Durrington, P.N. (1991) Substrate specificity of human serum paraoxonase. Biochem. Soc. Trans. 19, 304S.

[4] Playfer, J.R., Eze, L.C., Bullen, M.F. and Evans, D.A.P. (1976) Genetic polymorphism and interethnic variability of plasma paraoxonase activity. J. Med. Genet. 13, 337–342.

[5] Furlong, C.E., Richter, R.J., Seidel, S.L., Costa, L.G. and Motulsky, A.G. (1989) Spectrophotometric assays for the enzymatic hydrolysis of the active metabolites of chlorpyrifos and parathion by plasma paraoxonase/arylesterase. Anal. Biochem. 180, 242–247.

[6] Furlong, C.E., Costa, L.G., Hassett, C., Richter, R.J., Sundstrom, J.A., Adler, D.A., Disteche, C.M., Omiecinski, C.J., Chapline, C., Crabb, J.W. and Humbert, R. (1993) Human and rabbit paraoxonases: purification, cloning, sequencing, mapping and role of polymorphism in organophosphate detoxification. Chem.-Biol. Interact. 87, 35–48.

[7] Furlong, C.E., Richter, R.J., Chapline, C. and Crabb, J.W. (1991) Purification of rabbit and human serum paraoxonase. Biochemistry 30, 10133–10140.

[8] Gan, K.H., Smolen, A., Eckerson, H.W. and La Du, B.N. (1991) Purification of human serum paraoxonase/arylesterase — evidence for one esterase catalyzing both activities. Drug Metab. Dispos. 19, 100–106.

[9] Mackness, M.I., Hallam, S.D., Peard, T., Warner, S.M. and Walker, C.H. (1985) The separation of sheep and human serum A-esterase activity into the lipoprotein fraction by ultracentrifugation. Comp. Biochem. Physiol. 82B(4), 675–677.

[10] Hassett, C., Richter, R.J., Humbert, R., Chapline, C., Crabb, J.W., Omiecinski, C.J. and Furlong, C.E. (1991) Characterization of cDNA clones encoding rabbit and human serum paraoxonase: the mature protein retains its signal sequence. Biochemistry 30, 10141–10149.

[11] Adkins, S., Gan, K.N., Mody, M. and La Du, B.N. (1993) Molecular basis for the polymorphic forms of human serum paraoxonase/arylesterase: glutamine or arginine at position 191, for the respective A or B allozymes. Am. J. Hum. Genet. 52, 598–608.

[12] Humbert, R., Adler, D.A., Disteche, C.M., Hassett, C., Omiecinski, C.J. and Furlong, C.E. (1993) The molecular basis of the human serum paraoxonase activity polymorphism. Nat. Genet. 3, 73–76.

[13] Eckerson, H.W., Wyte, C.M. and La Du, B.N. (1983) The human paraoxonase/arylesterase polymorphism. Am. J. Hum. Genet. 35, 1126–1138.

[14] Omenn, G.S. (1987) The role of genetic differences in human susceptibility to pesticides. In: L.G. Costa, C.L. Galli and S.D. Murphy (Eds.), Toxicology of Pesticides: Experimental, Clinical and Regulatory Perspectives, Springer-Verlag, Heidelberg, pp. 93–107.

[15] Mutch, E., Blain, P.G. and Williams, F.M. (1992) Interindividual variations in enzymes controlling organophosphate toxicity in man. Hum. Exp. Toxicol. 22, 109–116.

[16] Brealey, C.J., Walker, C.H. and Baldwin, B.C. (1980) A-esterase activities in relation to the differential toxicity of pirimiphos-methyl to birds and mammals. Pestic. Sci. 11, 546–554.

[17] Costa, L.G., Richter, R.J., Murphy, S.D., Omenn, G.S., Motulsky, A.G. and Furlong, C.E. (1987) Species differences in serum paraoxonase correlate with sensitivity to paraoxon toxicity. In: L.G. Costa, C.L. Galli and S.D. Murphy (Eds.), Toxicology of Pesticides: Experimental, Clinical and Regulatory Perspectives, Springer Verlag, Heidelberg, pp. 263–266.

[18] Main, A.R. (1956) The role of A-esterase in the acute toxicity of paraoxon, TEPP and parathion. Can. J. Biochem. Physiol. 34, 197–216.

[19] Costa, L.G., McDonald, B.E., Murphy, S.D., Omenn, G.S., Richter. R.J., Motulsky, A.G. and Furlong, C.E. (1990) Serum paraoxonase and its influence on paraoxon and chlorpyrifos-oxon toxicity in rats. Toxicol. Appl. Pharmacol. 103, 66–76.

[20] Furlong, C.E., Richter, R.J., Seidel, S.L. and Motulsky, A.G. (1988) Role of genetic polymorphism of human plasma paraoxonase/arylesterase in hydrolysis of insecticide metabolites chlorpyrifos oxon and paraoxon. Am. J. Hum. Genet. 43, 230–238.

[21] Ellman, G.L., Courtney, K.D., Andres, V. Jr. and Featherstone, R.M. (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95.

[22] Benke, G.M., Cheever, K.L., Mirer, F.E. and Murphy, S.D. (1974) Comparative toxicity, anti-cholinesterase action and metabolism of methylparathion and parathion in sunfish and mice. Toxicol. Appl. Pharmacol. 28, 97–109.

[23] Voss, G. and Sachsse, K. (1970) Red cell and plasma cholinesterase activities in microsamples of human and animal blood determined simultaneously by a modified acetylthiocholine/DTNB procedure. Toxicol. Appl. Pharmacol. 16, 764–772.

[24] Altman, D.G. (1991) Practical Statistics for Medical Research. Chapman and Hall, London, pp. 191–194.

[25] Li, W.F., Costa, L.G. and Furlong, C.E. (1993) Serum paraoxonase status: a major factor in determining resistance to organophosphates. J. Toxicol. Environ. Health 40, 337–346.

[26] Kaliste-Korhonen, E., Ylitalo, P., Hanninen, O. and Raushel, F.M. (1993) Phosphotriesterase decreases paraoxon toxicity in mice. Toxicol. Appl. Pharmacol. 121, 275–278.

[27] Doctor, B.P., Raveh, L., Wolfe, A.D., Maxwell, D.M. and Ashani, Y. (1991) Enzymes as pretreatment drugs for organophosphate toxicity. Neurosci. Biobehav. Rev. 15, 123–128.

[28] Wolfe, A.D., Rush, R.S., Doctor, B.P., Kiplovitz, I. and Jones, D. (1987) Acetylcholinesterase prophylaxis against organophosphate toxicity. Fundam. Appl. Toxicol. 9, 266–270.

[29] Ashani, Y., Rothschild, N., Segall, Y., Levanon, D. and Raveh, L. (1991) Prophylaxis against organophosphate poisoning by an enzyme hydrolysing organophosphorus compounds in mice. Life Sci. 49, 367–374.

EXHIBIT 3

The effect of the human serum paraoxonase polymorphism is reversed with diazoxon, soman and sarin

Holly G. Davies[1], Rebecca J. Richter[1,2], Matthew Keifer[3], Clarence A. Broomfield[4], Jason Sowalla[4] & Clement E. Furlong[1,2,5]

Many organophosphorus compounds (OPs) are potent cholinesterase inhibitors, accounting for their use as insecticides and, unfortunately, also as nerve agents. Each year there are approximately 3 million pesticide poisonings world-wide resulting in 220,00 deaths[1–2]. In 1990, there were 1.36 million kg of chlorpyrifos, 4.67 million kg of diazinon and 1.23 million kg of ethyl parathion manufactured in the USA (data supplied by the USEPA). In addition to exposure risks during pesticide manufacturing, distribution and use, there are risks associated with the major international effort aimed at destroying the arsenals of nerve agents, including soman and sarin. The United States has pledged to destroy approximately 25,000 tons of chemical agents by the end of the decade[3]. The high density lipoprotein (HDL)-associated enzyme paraoxonase (PON1) contributes significantly to the detoxication of several OPs (Fig. 1). The insecticides parathion, chlorpyrifos and diazinon are bioactivated to potent cholinesterase inhibitors[4] by cytochrome P-450 systems[5]. The resulting toxic oxon forms can be hydrolysed by PON1, which also hydrolyses the nerve agents soman and sarin[6] (Fig. 1). PON1 is polymorphic in human populations and different individuals also express widely different levels of this enzyme[7–9]. The Arg_{192} (R_{192}) PON1 isoform hydrolyses paraoxon rapidly, while the Gln_{192} (Q_{191}) isoform hydrolyses paraoxon slowly[6,10]. Both isoforms hydrolyse chlorpyrifos-oxon[8,9] and phenylacetate[6,7,9] at approximately the same rate. The role of PON1 in OP detoxication is physiologically significant[11–15]. Injected PON1 protects against OP poisoning in rodent model systems[12–15] and interspecies differences in PON1 activity correlate well with observed [redacted] an lethal dose (LD_{50}) values[8,11,16]. We report here a simple enzyme analysis that provides a clear resolution of PON1 genotypes and phenotypes allowing for a reasonable assessment of an individual's probable susceptibility or resistance to a given OP, extending earlier studies on this system. We also show that the effect of the PON1 polymorphism is reversed for the hydrolysis of diazoxon, soman and especially sarin, thus changing the view of which PON1 isoform is considered to be protective.

In the course of evaluating the PON1 status of farm workers prior to pesticide exposure during the growing season, we also determined the rates of diazoxon hydrolysis. By plotting the activity distributions for the three substrates, chlorpyrifos oxon, phenylacetate and diazoxon, against the rates of paraoxon hydrolysis, we were able to clearly resolve individuals homozygous for the low-activity paraoxonase isoform (QQ individuals) from heterozygotes (QR individuals) (Fig. 2*a–c*). However, only the plot of diazoxon hydrolysis versus paraoxon hydrolysis (Fig. 2*c*) clearly resolved all three genotypes and at the same time provided important information about the level of enzyme expressed in a given individual. This two-dimensional enzyme analysis provides a complete assessment of an individual's PON1 status (genotype and phenotype). PON1 levels in a given individual are usually very stable over time[17].

One of the most interesting observations was the reversal of the effect of the PON1 activity polymorphism for diazoxon hydrolysis relative to paraoxon hydrolysis (Fig. 2*c*). RR homozygotes (high paraoxonase activity) had lower diazoxonase activities (mean=7948 U/l) than QQ homozygotes (mean=12,318 U/l). Average rates of diazoxon hydrolysis (10,619 U/l) were somewhat higher than the rates of chlorpyrifos oxon hydrolysis (8233 U/l), suggesting that on average, humans may be better able to detoxicate diazinon than chlorpyrifos or parathion.

We also observed an increased frequency for the R_{192} allele (0.41) in this Hispanic population compared with a frequency of 0.31 observed in populations of Northern European origin[18]. This results in approximately 16% of individuals of Hispanic origin being homozygous for the R_{192} PON1 isoform compared with only 9% of individuals of Northern European origin[8,18].

Following the March 1995 release of sarin in the Tokyo subway, we examined the effect of the PON1 polymorphism on soman and sarin hydrolysis, as PON1 is the only enzyme from humans known to hydrolyse the phosphorus-fluorine bond of these very toxic nerve agents[6]. It is clear that the effect of the polymorphism is reversed for both of these compounds, especially sarin (Fig. 2*d, e*). The mean value for sarin hydrolysis was only 38 U/l for the R_{192} homozygotes compared with 355 U/l for the Q_{192} homozygotes (Table 1). The ranges of values for hydrolysis of each of the PON1 substrates are also shown in Table 1.

These results help to explain the large individual differences in sensitivity to OP insecticides processed through the P-450/PON1 pathway or hydrolysed directly by PON1. As the dose response curves for OP toxicity are very steep[12], a small percentage difference in metabolic rate can represent a significant difference in OP sensitivity. In this light, it is important to note that we found in earlier studies that newborns have very low levels of PON1[19], leading to the prediction that newborns are probably significantly more sensitive than adults to OP compounds processed by PON1. Increased sensitivity to OPs has been observed in newborn rats[20,21].

In addition to playing a major role in OP detoxication, the PON1 polymorphism has been recently implicated in another important area of human health. Watson *et al.*[22] demonstrated that PON1 destroys biologically oxidized phospholipids, while other investigators have shown that the R_{192} allele represents a risk factor for coronary artery disease[23,24]. These studies suggest that the same considerations given to the determination of both PON1 genotype and phenotype (PON1 status) relative to OP sensitivity will also be important for studies on the role of PON1 in vascular disease.

These studies underline the importance of examining the effects of polymorphisms on each substrate or

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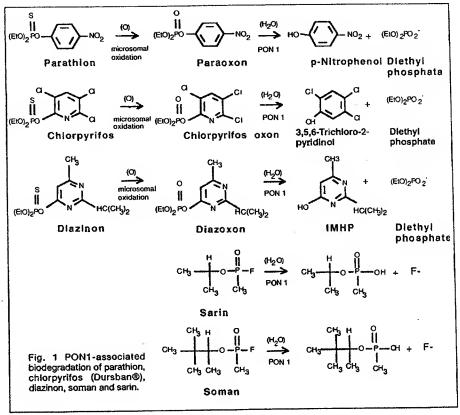

Fig. 1 PON1-associated biodegradation of parathion, chlorpyrifos (Dursban®), diazinon, soman and sarin.

inhibitor of physiological importance. A single amino acid mutation in acetylcholinesterase has been demonstrated to cause a reversal in sensitivities of leaf hoppers to specific OP insecticides[25]. Reversal of sensitivity to inhibitors by single amino acid changes have also been observed in plant[26] and viral[27] systems. The effect of the PON1 polymorphism on sarin hydrolysis illustrates how dramatic the reversal of the effect of an enzyme polymorphism can be.

Methods

Human subjects. Plasma (heparin) from 92 individuals of Hispanic origin were drawn via venipuncture with informed consent.

Enzyme assays. Hydrolysis rates of paraoxon[8], phenylacetate[28] and chlorpyrifos oxon (CPO)[8] were determined as described. Rates of diazoxon hydrolysis were determined by a continuous spectrophotometric assay developed in our laboratory (R.J.R. and C.E.P., manuscript in preparation) based on published

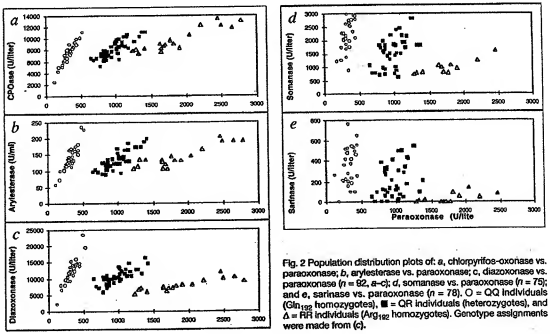

Fig. 2 Population distribution plots of: *a*, chlorpyrifos-oxonase vs. paraoxonase; *b*, arylesterase vs. paraoxonase; *c*, diazoxonase vs. paraoxonase (*n* = 92, *a*–*c*); *d*, somanase vs. paraoxonase (*n* = 75); and *e*, sarinase vs. paraoxonase (*n* = 78). O = QQ individuals (Gln_{192} homozygotes), ■ = QR individuals (heterozygotes), and Δ = RR individuals (Arg_{192} homozygotes). Genotype assignments were made from (*c*).

Table 1 Ranges of PON substrate activities in human serum

	Diazoxonase[a] (U/L)		Sarinase[a] (U/L)		Somanase[a] (U/L)	
	Range	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.
All	2174–23316[b]	10619 ± 3207	0–756[c]	230 ± 191	616–2982[d]	1658 ± 660
QQ	2174–23316[e]	12318 ± 3748	0–758[f]	355 ± 183	870–2982[g]	2143 ± 576
QR	5903–16277[h]	10426 ± 2302	0–541[i]	198 ± 161	616–2815[j]	1518 ± 558
RR	5400–11193[k]	7948 ± 1712	0–144[l]	38 ± 47	754–1616[m]	992 ± 263

	Paraoxonase[a] (U/L)		CPOase[a] (U/L)		Arylesterase[a] (U/ml)	
	Range	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.
All	121–2786[b]	924 ± 603	2415–13540[b]	8233 ± 1908	57–235[b]	136 ± 32
QQ	121–532[e]	326 ± 79	2415–11101[e]	7484 ± 1640	57–235[e]	138 ± 37
QR	593–1418[h]	977 ± 171	5134–11160[h]	8152 ± 1519	88–198[h]	131 ± 28
RR	1237–2786[k]	1769 ± 354	7480–13540[k]	9794 ± 2001	106–205[k]	145 ± 32

[a]Assays are described in Methods. [b]$n = 92$, [c]$n = 78$, [d]$n = 73$, [e]$n = 28$, [f]$n = 26$, [g]$n = 41$, [h]$n = 38$, [i]$n = 36$, [j]$n = 18$, [k]$n = 12$, [l]$n = 11$. All = all individuals in study, QQ = Gln_{191} homozygotes, QR = heterozygotes, RR = Arg_{191} homozygotes.

spectral data[29,30]. The incubation mixtures contained 0.1 M Tris-HCl, pH 8.5, 2.0 M NaCl, 2.0 mM $CaCl_2$, 500 μM diazoxon, and 5 μl of plasma in a volume of 1 ml at 24 °C. Appearance of 2-isopropyl-4-methyl-6-hydroxy pyrimidine (IMHP) was continuously monitored at 270 nm in a Beckman DU-70 spectrophotometer. The reaction was initiated by addition of plasma.

Hydrolysis rates of sarin and soman were determined at the USAMRICD Facility with a titrimetric procedure, using a Radiometer TTT80 pH-stat and an ABU80 autoburette. 3 ml of 1 mM soman or sarin in 1.0 M NaCl with 2.0 mM $CaCl_2$ were added to a temperature-controlled reaction vessel fitted with a capillary delivery tube from the autoburette, and the background hydrolysis rate was monitored for several minutes. Then, 50 μl of plasma were added and the resulting hydrolysis rate monitored at 25 °C. The background rate was subtracted from the sample hydrolysis rate. All samples were measured in triplicate.

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1. World Health Organization. Informal consultation on planning strategy for the prevention of pesticide poisoning. Geneva, 25–29 November 1985. WHO/VBC/86.926. (Geneva: WHO, 1986).
2. World Health Organization. Public health impact of pesticides used in agriculture. (Geneva: WHO, 1990).
3. U.S. Congress, Office of Technology Assessment. *Disposal of Chemical Weapons: Alternative Technologies — Background Paper*. OTA-BP-O-95 (Washington, DC: U.S. Government Printing Office, June 1992).
4. Tafuri, J. & Roberts, J. Organophosphate poisoning. *Ann. Emerg. Med.* **16**, 193–202 (1987).
5. Murphy, S.D. in *Toxicology: The Basic Science of Poisons*. (eds Doull, J., Klassen, C., & Amdur, M.) 357–408 (Macmillan, New York, 1980).
6. Smolen, A., Eckerson, H.W., Gan, K.N., Hallet, N. & LaDu, B.N. Characteristics of the genetically determined allozymic forms of human serum paraoxonase/arylesterase. *Drug Metab. Dispos.* **19**, 107–112 (1991).
7. LaDu, B.N., Pike, J.E., Eckerson, H.W., Vincent-Vigy, M. & Selat, G. An improved method for phenotyping individuals for the human serum paraoxonase arylesterase polymorphism. *Ann. Biol. Clin.* **44**, 369–372 (1986).
8. Furlong, C.E., Richter R.J., Seidel, S.L., Costa, L.G. & Motulsky, A.G. Spectrophotometric assays for the enzymatic hydrolyses of the active metabolites of chlorpyrifos and parathion by plasma paraoxonase/arylesterase. *Analyt. Biochem.* **180**, 242–247 (1989).
9. Furlong, C.E., Richter, R.J., Seidel, S.L., Costa, L.G. & Motulsky, A.G. Role of genetic polymorphism of human plasma paraoxonase/arylesterase in hydrolysis of the insecticide metabolites chlorpyrifos oxon and paraoxon. *Am. J. Hum. Genet.* **43**, 230–238 (1988).
10. Humbert, R. *et al*. The molecular basis of the human serum paraoxonase activity polymorphism. *Nature Genet.* **3**, 73–76 (1993).
11. Costa, L.G. *et al*. Species differences in serum paraoxonase activity correlate with sensitivity to paraoxon toxicity. *Toxicology of Pesticides: Experimental, Clinical and Regulatory Aspects*, in NATO ASI Series, Vol. H13. (Eds., L. G. Costa, *et al.*) 263–266 (Springer-Verlag, Berlin 1987).
12. Costa, L.G. *et al*. Serum paraoxonase and its influence on paraoxon and chlorpyrifos-oxon toxicity in rats. *Tox. Appl. Pharmac.* **103**, 66–76 (1990).
13. Li, W.-F., Costa, L.G. & Furlong, C.E. Serum paraoxonase status: a major factor in determining resistance to organophosphates. *J. Tox. Envir. Hlth* **40**, 337–346 (1993).
14. Li, W.-F., Furlong, C. E. & Costa, L.G. Paraoxonase protects against chlorpyrifos toxicity in mice. *Tox. Lett.* **76**, 219–226 (1995).
15. Main, A.R. The role of A-esterase in the acute toxicity of paraoxon, TEPP, and parathion. *Can. J. Biochem.* **34**, 197–216 (1956).
16. McCollister, S.B., Kociba, R.J., Humiston, C.G. & McCollister, D.D. Studies on the acute and long-term oral toxicity of chlorpyrifos (0,0-diethyl-0(3,5,6-trichloro-2-pyridyl) phosphorothioate). *Food Cosmet. Tox.* **12**, 45–61 (1974).
17. Zech, R. & Zurcher, K. Organophosphate splitting enzymes in different mammals. *Comp. Biochem. Physiol.* **49B**, 427–433 (1974).
18. Geldmacher-von Mallinckrodt, M. & Deegen, T.L. The human serum paraoxonase-polymorphism and specificity. *Toxicol. Environ. Chem.* **18**, 79–196 (1988).
19. Mueller, R.F. *et al*. Plasma paraoxonase polymorphism: a new enzyme assay, population, family, biochemical and linkage studies. *Am. J. Hum. Genet.* **36**, 393–408 (1983).
20. Benke G.M. & Murphy S.D. The influence of age on the toxicity and metabolism of methyl parathion and parathion in male and female rats. *Tox. Appl. Pharmac.* **31**, 254–269 (1975).
21. Burnett W.T. & Chambers J.E. Age-related toxicity of the insecticides parathion and chlorpyrifos. *Toxicologist* **14**, 390 (1994).
22. Watson, A.D. *et al*. Protective effect of high density lipoprotein associated paraoxonase: inhibition of the biological activity of minimally oxidized low density lipoprotein. *J. Clin. Invest.* **96**, 2882–2891 (1995).
23. Ruiz, J. *et al*. Gln-Arg192 polymorphism of paraoxonase and coronary heart disease in type 2 diabetes. *Lancet.* **346**, 869–872 (1995).
24. Serrato, M. & Marian, A.J. A variant of human paraoxonase/arylesterase (HUMPONLA) gene is a risk factor for coronary artery disease. *J. Clin. Invest.* **96**, 3005–3008 (1995).
25. Hama, H., Iwata, T., Miyata, T. & Saito, T. Some properties of acetylcholinesterases partially purified from susceptible and resistant green rice leafhoppers, *Nephotettix cincticeps* Uhler (Hemiptera: Deltocephalidae). *Appl. Ent. Zool.* **18**, 249–261 (1983).
26. St.Clair, M.H. *et al*. Resistance to ddI and sensitivity to AZT induced by a mutation in HIV-1 reverse transcriptase. *Science* **253**, 1557–1559 (1991).
27. Arnzten, C.J., Pfister, K. & Steinback, K. The mechanism of chloroplast triazine resistance: alterations in the site of herbicide action. In *Herbicide Resistance in Plants* (eds LeBaron, H.M. & Gressel, J.) 194–195 (John Wiley & Sons, New York, 1982).
28. Kitchen, G.J., Masters, C.J. & Winzor, D.J. Effects of lipid removal on the molecular size and kinetic properties of bovine plasma arylesterase. *Biochem. J.* **139**, 93–99 (1973).
29. Gomaa, H.M., Suffet, I.H. & Faust S.D. Kinetics of hydrolysis of diazinon and diazoxon. *Residue Rev.* **29**, 171–190 (1969).
30. Shishido, T. & Fukami, J.-I. Enzymatic hydrolysis of diazoxon by rat tissue homogenates. *Pestic. Biochem. Physiol.* **2**, 39–50 (1972).

EXHIBIT 4

US005721118A

United States Patent [19]

Scheffler

[11] Patent Number: 5,721,118

[45] Date of Patent: Feb. 24, 1998

[54] MAMMALIAN ARTIFICIAL CHROMOSOMES AND METHODS OF USING SAME

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[73] Assignee: The Regents of the University of California, San Diego, Alameda, Calif.

[21] Appl. No.: 741,406

[22] Filed: Oct. 29, 1996

Related U.S. Application Data

[60] Provisional application No. 60/039,256, Oct. 31, 1995.

[51] Int. Cl.[6] C12N 15/06; C12N 15/12; C12N 15/16; C12N 5/28

[52] U.S. Cl. 435/69.1; 435/172.2; 435/172.3; 435/320.1; 435/325; 514/44; 800/2; 536/23.1; 536/23.5; 935/11; 935/89; 935/90; 935/92; 935/93; 935/95; 935/96; 935/106; 935/107

[58] Field of Search 435/320.1, 325, 435/172.2, 172.3, 69.1; 514/44; 800/2; 536/23.1, 23.5; 935/11, 89, 90, 92, 93, 95, 96, 106, 107

[56] References Cited

U.S. PATENT DOCUMENTS

5,288,625 2/1994 Hadlaczky 435/172.2

OTHER PUBLICATIONS

Carine et al., "Chinese Hamster Cells with a Minichromosome Containing the Centromere Region of Human Chromosome 1" *Somat. Cell Mol. Gen.* 12:479–491 (1986).

Carine et al., "Molecular Characterization of Human Minichromosomes with Centromere from Chromosome 1 in Human Hamster Hybrid Cells" *Somat. Cell Mol. Gen.* 15:445–460 (1989).

Cochran et al., "The cDNA Sequence of Beef Heart CII–3, a Membrane–intrinsic Subunit of Succinate–ubiquinone Oxidoreductase" *Biochemica et Biophysica Acta* 1188:162–166 (1994).

Farr et al., "Generation of a Human X–derived Minichromosome Using Telomere–associated Chromosome Fragmentation" *EMBO J.* 14:5444–5454 (1995).

Heller et al., "Mini–chromosomes derived from the human Y chromosome by telomere directed chromosome breakage" *Proc. Nat'l Acad. Sci. USA*, 93:7125–7130 (1996).

Huxley et al., "Ordering up Big MACS" *Bio/Technology* 12:586–590 (1994).

Huxley, "Transfer of YAC's to Mammalian Cells and Transgenic Mice" *Genetic Engineering* 16:65–91 (1994).

Jakobovits et al., "Germ–line Transmission and Expression of a Human–derived Yeast Artificial Chromosome" *Nature* 362:255–258 (1993).

Mascarello et al., "Assignment of a Gene for Succinate Dehydrogenase to Human Chromosome 1 by Somatic Cell Hybridization" *Cyotogenetics Cell Genet.* 28:121–135 (1980).

Montoliu et al., "Germ Line Transmission of Yeast Artificial Chromosomes in Transgenic Mice" *Reprod. Fertility & Devel.* 6:577–584 (1994).

Oostveen et al., "A Chinese Hamster Mutant Cell Line with a Defect in the Integral Membrane Protein CII–3 of Complex II of the Mitochondrial Electron Transport Chain" *J. Biol. Chem.* 270:26104–26108 (1995).

Solus et al., "Characterization of Single–Copy Probe from Vicinity of Centromere of Human Chromosome 1" *Somat. Cell Mol. Genet.* 14: 381–391 (1988).

Willard, Huntington F., "Chromosome manipulation: A systematic approach toward understanding human chromosome structure and function" *Proc. Nat'l Acad. Sci. USA* 93:6847–6850 (1996).

Brown et al., Current Opinion in Genetics and Development, vol. 6, pp. 281–288, 1996.

Henikoff, Trends in Genetics, vol. 6, pp. 422–426, Dec. 1990.

Monaco et al., Trends in Biotechnology, vol. 12, pp. 280–286, Jul. 1994.

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[57] ABSTRACT

The present invention provides a mammalian artificial chromosome (MAC), comprising a centromere and a unique cloning site, said MAC containing less than 0.1% of the DNA present in a normal haploid genome of the mammalian cell from which the centromere was obtained. The invention further provides a MAC, wherein the unique cloning site is a nucleic acid sequence encoding a selectable marker. The invention also provides methods of preparing a MAC. In addition, the invention provides methods of stably expressing a selectable marker in a cell, comprising introducing a MAC containing the selectable marker into the cell. The invention also provides a cell containing a MAC expressing an exogenous nucleic acid sequence and a transgenic mammal expressing a selectable marker.

17 Claims, 3 Drawing Sheets

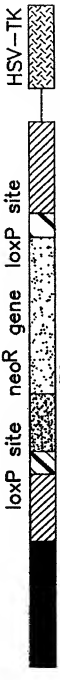

FIG. IA

FIG. IB

FIG. IC

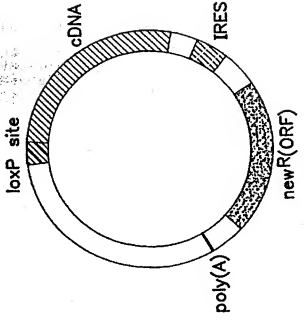
loxP site
cDNA
IRES
newR(ORF)
poly(A)

FIG. ID

SDH-3 Promoter
loxP site
cDNA
ATG
IRES
newR(ORF)
ATG
TAG
poly(A)
loxP site

FIG. IE

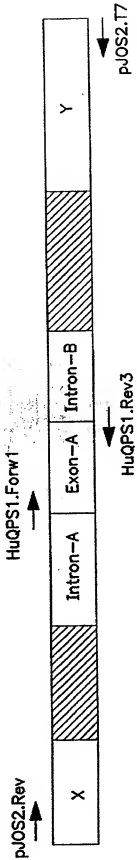
pJOS2.T7
Y
Intron–B
HuQPS1.Rev3
Exon–A
HuQPS1.Forw1
Intron–A
X
pJOS2.Rev

FIG. 2

MAMMALIAN ARTIFICIAL CHROMOSOMES AND METHODS OF USING SAME

This invention was made with government support under GM 23241 and GM 18835 awarded by the United States Public Health Service. The government has certain rights in this invention.

This application claims the benefit of priority of U.S. Provisional Application No. 60/039.256, filed Oct. 31, 1995, which was converted from U.S. Ser. No. 08/550,717, now abandoned, the entire contents of which is incorporated herein by reference.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates generally to molecular biology and molecular genetics and more specifically to mammalian artificial chromosomes.

2. Background Information

The ability to clone and express nucleic acid molecules has resulted in the identification of numerous genes and gene products. As a result of the identification of various genes, molecular differences between normal and diseased conditions are beginning to be recognized. For example, in various disease conditions such as Duchenne muscular dystrophy (DMD), cystic fibrosis and some forms of cancer, mutations in particular genes appear to be the basis for the underlying pathology. In DMD, for example, a mutation in the dystrophin gene can result in the formation of only part of the dystrophin protein, which functions abnormally and contributes to the characteristic signs and symptoms of DMD.

The identification of a molecular defect as the cause of a particular disease suggests possible approaches for ameliorating the disease at the molecular level. Gene therapy, in particular, holds the promise of correcting a pathology such as DMD by introducing a normal dystrophin gene into the muscle cells of an individual suffering from DMD. Unfortunately, the specific molecular defect has been identified in only a handful of diseases. In addition, some genes such as the dystrophin gene contain over one million base pairs and, therefore, are too large to be conveniently transferred from one cell into another using currently available technology.

It has been proposed that the identification of every gene in the human genome will provide insight into the mechanisms responsible for many diseases. Thus, the Human Genome Project was initiated to develop a linkage map for each of the twenty-three pairs of human chromosomes and, ultimately, to obtain the nucleic acid sequence of the entire human genome. However, a structural description, alone, of the human genome is not likely to be sufficient to allow, for example, an understanding of the mechanisms of gene regulation, which can depend on DNA regulatory elements that are located thousands of base pairs or more from the regulated genes.

Currently available mammalian vectors such as retrovital vectors can harbor, at best, DNA fragments containing up to about ten thousand nucleotides. In comparison, yeast vectors such as yeast artificial chromosomes (YACs) can harbor DNA fragments having a few hundred thousand nucleotides. However, such YAC vectors are not stable in mammalian cells and, therefore, cannot be used, for example, as vectors for gene therapy, which, ideally, would be stably maintained in a cell from generation to generation and would express a predictable amount of a gene product. Thus, a need exists for vectors that can contain large fragments of DNA and that are stably maintained in mammalian cells. The present invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

The present invention provides a mammalian artificial chromosome (MAC), comprising a centromere and unique cloning site, said MAC containing less than about one-tenth of one percent (0.1%) of the DNA present in a normal haploid mammalian genome from which the centromere was obtained. The invention further provides a MAC, wherein the unique cloning site is a nucleic acid sequence encoding a selectable marker. For example, the invention provides a MAC having a centromere from human chromosome I and a nucleic acid molecule encoding a subunit (designated CII-3) of complex II of the mitochondrial electron transport chain, wherein the MAC contains less than about 0.05% of the DNA normally present in a haploid human genome.

The invention also provides methods of using a MAC. For example, the invention provides methods of stably expressing an exogenous nucleic acid molecule in a cell, comprising introducing a MAC containing the exogenous nucleic acid molecule into the cell. The invention also provides a cell containing a MAC expressing an exogenous nucleic acid sequence and a transgeaic mammal expressing a selectable marker.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 illustrates targeting vectors for introducing a loxP site, which is a target sequence recognized by the bacteriophage P1Cre recombinase, into MAC-8.2.3. FIG. 1A shows a targeting vector containing two loxP sites ("loxP site") flanking the neomycin resistance gene ("neo^R gene"), which contains the ATG start codon and the neogene promoter (located between the 5' loxP site and the ATG sequence). Flanking the loxP sites are genomic DNA sequences of the human CII-3 gene; the darkly stippled bar indicates the CII-3 promoter and the hatched bars indicate CII-3 gene exon or intron sequences. Outside of the CII-3 gene sequences is the HSV-tk gene, which is lost following homologous recombination of the vector into the CII-3 gene present on MAC-8.2.3 (see FIG. 1B).

FIG. 1B shows the targeting vector of FIG. 1A following homologous recombination into the CII-3 gene present on MAC-8.2.3.

FIG. 1C shows the integration site as illustrated in FIG. 1B following excision of the neo gene by the Cre recombinase. Following Cre recombinase action, a single loxP site remains in MAC-8.2.3.

FIG. 1D illustrates a second targeting vector, which can insert into a loxP site such as that shown in FIG. 1C. The circularized vector contains a single loxP site, which can insert into a loxP site present in a MAC due to Cre recombinase activity. The vector contains an exogenous nucleic acid sequence ("cDNA") and the promoterless open reading frame encoding the neo gene product ("neo^R(ORF)"). Following insertion of the vector into a loxP site such as that shown in FIG. 1C, a dicistronic transcript (cDNA-neo) is produced; expression of the transcript is from the CII-3 promotor present in MAC-8.2.3 (see FIG. 1E). The construct also contains an internal ribosome entry site ("IRES"), which allows translation of the neo open reading frame in the dicistronic transcript, and a polyadenylation site ("poly (A)"), which allows polyadenylation of the dicistronic transcript.

FIG. 1E shows the vector of FIG. 1D after insertion into the loxP site of FIG. 1C. The CII-3 promoter is shown ("SDH-3 promoter). Initiator methionine codons ("ATG") and STOP codons ("TAG") also are shown.

FIG. 2 provides a schematic representation of the genomic CII-3 DNA sequence cloned in pJOS2. "Intron-A" (SEQ ID NO: 3), "Exon-A" (SEQ ID NO: 4) and "Intron-B" (SEQ ID NO: 5) are indicated. "X" (SEQ ID NO: 6) and "Y" (SEQ ID NO: 7) also are indicated. "pJOS2.Rev" (SEQ ID NO: 8). "pJOS2.T7" (SEQ ID NO: 9). HuQPS1.Forw1" (SEQ ID NO: 10) and "HuQPS1.Rev3" (SEQ ID NO: 11) indicate the location of primers and the associated arrows indicate the direction of synthesis from the primer. "Hatching" indicates that the length and sequence of the genomic DNA has not yet been determined. The subclone is shown with the 5'-end at the left and the 3'-end at the left, with respect to the coding sequence of Exon-A (see SEQ ID NO: 1), and is approximately to scale, except for size and position of the hatched regions.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a mammalian artificial chromosome (MAC), comprising a centromere and a unique cloning site, said MAC containing less than 0.1% of the DNA present in a haploid mammalian genome normally containing the centromere. The invention further provides a MAC, wherein the unique cloning site is a nucleic acid sequence encoding a selectable marker. The invention provides, for example, a MAC containing a portion of human chromosome 1, including the human chromosome 1 centromere and the CII-3 gene, which encodes a subunit (CII-3) of complex II of the mitochondrial electron transport chain and allows survival of SDH defective hamster cells in glucose-free medium (Mascarello et al., *Cytogenet. Cell Genet.* 28:121–135 (1980), and Carine et al., *Somat. Cell Genet.* 12:479–492 (1986), each which is incorporated herein by reference). The CII-3 gene encodes one subunit of the complex known as complex II of the mitochondrial electron transport chain. As disclosed herein, a mutation in the CII-3 gene results in deficient succinate dehydrogenase (SDH) activity in a hamster cell line. Thus, reference is made herein to "SDH deficient cells" or "respiration deficient cells" and the like with the understanding that the deficiency is due to a defect of the CII-3 subunit of complex II.

As used herein, the term "mammalian artificial chromosome" or "MAC" means a nucleic acid molecule that 1) forms a centromere, 2) contains an origin of DNA replication, and 3) has a unique cloning site, wherein the size of the MAC, excluding the centromere, is less than about 0.1% of the size of a haploid mammalian genome normally containing the centromere present in the MAC. In particular, a MAC of the invention contains a nucleic acid sequence encoding a selectable marker, which can be used as a site into which an exogenous nucleic acid sequence can be cloned. Due, in part, to the structural characteristics described above, a MAC is characterized further in that it is stably and autonomously maintained in a host cell and, therefore, is present in both daughter host cells following mitosis of the parental host cell. A MAC can be prepared from any mammalian chromosome, including a human, murine, bovine, ovine, porcine or other mammalian chromosome such as human chromosome 1 as exemplified herein.

A Chinese hamster cell line, XEW8.2.3, which is a host cell for a MAC having a human chromosome 1 centromere and the human CII-3 gene and containing less than about 0.05% of the DNA in a normal haploid human genome, has been deposited in accordance with the requirements of the Budapest Treaty with the American Type Culture Collection (ATCC) on Oct. 31, 1995, as ATCC Accession No: ATCC CRL 11992. For convenience, the MAC present in the cell line available as ATCC Accession No: ATCC CRL 11992 is referred to herein as MAC-8.2.3.

A MAC of the invention is defined, in part, by having a size, excluding the centromere, that is less than about 0.1% of DNA present in a normal mammalian haploid genome, the particular mammalian genome being that genome from which the centromere is obtained. For example, MAC-8.2.3 is defined in terms of a human genome because the centromere of MAC-8.2.3 was obtained from human chromosome 1. A human haploid genome contains about 3.3×10^9 base pairs of DNA. Thus, a MAC having a centromere obtained from a human genome contains a centromere and less than about 3.3 million base pairs. MAC-8.2.3, for example, contains a centromere and, in addition, about 1–2 million base pairs of DNA on the arms flanking the centromere. It is recognized, however, that, while a MAC is defined as having a size that is less than about 0.1% of a normal mammalian haploid genome, the MAC can be used as a vector and, therefore, can contain inserted DNA sequences that can be several million base pairs in size. Methods for estimating the size of a putative MAC are known in the art (see, for example, Carine et al., supra, 1986).

As used herein, the term "less than 0.1% of the DNA present in a haploid mammalian genome normally containing the centromere" refers to the amount of DNA that contains repetitive DNA sequences known as Alu sequences. In general, Alu-containing DNA is considered to be present in the chromosomal arm, but not in the centromeres. Thus, a MAC as defined herein, contains, in addition to a centromere, less than about 3.3×10^6 base pairs of DNA on the arms flanking the centromere. The amount of DNA in a MAC that contains Alu sequences can be estimated using methods well known in the art (see, for example, Carine et al., supra, 1986).

As used herein, the term "centromere" means the DNA sequence that normally is present at the junction between the two arms of a chromosome and is associated with the structure to which the spindle fibers attach during mitosis. For purposes of the present invention, a centromere is identified by its function of providing stable segregation during cell division of a nucleic acid sequence linked to the centromere. While it is recognized that the spindle fibers likely do not attach directly to the DNA sequence contained in a centromere but, instead, attach to a nucleoprotein complex formed, in part, by the DNA sequence, no mechanism is proposed herein as to how a centromere functions. The term "centromeric fragment" is used herein to mean a portion of a chromosome containing a centromere. As disclosed herein, a centromeric fragment can be obtained, for example, by irradiating a cell at a dose that results in breakage of the chromosomes.

The skilled artisan would recognize that a MAC containing an origin of DNA replication can be identified by detecting the MAC in both daughter cells formed following mitosis of the parental host cell. The presence of a selectable marker in a MAC can be identified by determining that a cell containing the MAC has the characteristics conferred by the marker. Selectable markers are described in greater detail below.

As used herein, the term "host cell" is used broadly to mean a cell containing a MAC. XEW8.3.2 is an example of

a host cell. In general, a host cell is useful for maintaining a MAC and is a convenient "vessel" for manipulating the MAC. For example, an exogenous nucleic acid molecule can be introduced into a MAC by transfecting the host cell containing the MAC with the nucleic acid sequence under conditions that allow the exogenous sequence to be inserted into the MAC. An exogenous nucleic acid sequence can be inserted into MAC-8.2.3, for example, by homologous recombination of a targeting vector containing the sequence with the CII-3 gene present in MAC-8.2.3.

As used herein, the term "exogenous nucleic acid sequence" when used in reference to a MAC means a nucleotide sequence that is not normally present on the MAC. In contrast, the term "endogenous nucleic acid sequence" means a nucleotide sequence normally present on the MAC. Thus, the human CII-3 gene, which is normally present in a pericentric location on human chromosome 1, is an example of an endogenous nucleic acid sequence with reference to MAC-8.2.3 (see, for example, SEQ ID NOS: 3–7). Any other nucleic acid sequence that, for example, is inserted into the CII-3 gene sequence on MAC-8.2.3 is considered an exogenous nucleic acid sequence.

An exogenous nucleic acid sequence can be a fragment of genomic DNA, which can be prepared from intact genomic DNA by physical disruption using, for example, irradiation or sonication or by chemical cleavage using, for example, a restriction endonuclease such as a rare cutting endonuclease that cleaves genomic DNA at relatively few sites. A population of MACs containing diverse fragments of genomic DNA prepared from a particular cell type can constitute a genomic library, which can be screened, for example, to identify fragments containing particular genes of interest. Since a MAC can contain a fragment of genomic DNA having several million base pairs, such a genomic library can contain, for example, a complex genetic locus, thereby providing a model system useful for identifying the regulatory regions such as enhancers or silencers that are involved in regulation of gene expression from the locus and the regulatory factors that bind to such regions. Thus, a MAC is useful as a cloning vector and provides the additional advantage that very large fragments of DNA on the order of several million bases can be cloned into and maintained in the MAC.

An exogenous nucleic acid sequence also can be inserted into a MAC for the purpose of being expressed. Such an exogenous nucleic acid sequence can be, for example, a particular gene such as the gene encoding dystrophin; or can be a cDNA, which encodes a gene product; or can be a sequence that, when expressed, is complementary to a nucleic acid of interest and acts, for example, as an antisense molecule, which can hybridize to a particular DNA or RNA sequence, or acts as a ribozyme, which can hybridize to and cleave a particular RNA. Thus, a MAC also can be useful as an expression vector and provides the additional advantage that it is stable through numerous rounds of cell division.

An exogenous nucleic acid sequence also can include a regulatory element involved in the regulation of gene expression or of translation of a transcript. Such regulatory elements such as a promoter, enhancer, silencer, polyadenylation signal sequence, ribosome entry site, signal peptide encoding sequence, nuclear localization signal encoding sequence and the like are well known in the art and can be inserted into a MAC, as desired, using well known methods of recombinant DNA technology (see, for example, Kriegler, *Gene Transfer and Expression: A laboratory manual* (W.H. Freeman and Co., New York; 1990), which is incorporated herein by reference).

Various types of regulatory elements are available and are selected based on the particular purpose for which a MAC is being constructed. A promoter element, for example, can be constitutive such as the cytomegalovirus promoter or Rous sarcoma virus promoter, or can be inducible such as the metallothionein promotor. In addition, a promoter can be a tissue specific promotor such as the myoD promoter, which is expressed only in muscle cells, or the lck promoter, which is expressed only in T cells, or can be a promoter that is active only during a particular stage of development. Similarly, enhancers can be constitutive or inducible or, like the SV40 enhancer, can be constitutively active and, in addition, can be induced to a higher level of activity. Such gene regulatory elements and translation regulatory elements generally are relatively small and can be synthesized using routine methods of DNA synthesis or can be purchased in vectors from commercial sources.

A MAC is characterized, in part, by containing a unique cloning site. As used herein, the term "unique cloning site" means a nucleic acid sequence that can be targeted for insertion of an exogenous nucleic acid sequence. As disclosed herein, a unique cloning site can be, for example, a specific target site such as the loxP sequence, which is a target for the Cre recombinase, or an FLP site, which is a target for the FRP recombinase (see below). The presence of such a cloning site in a MAC allows the site specific integration of an exogenous nucleic acid sequence into the MAC.

A unique cloning site also can be a nucleic acid sequence encoding a gene product, provided the nucleic acid sequence is present in a single copy on the MAC. As disclosed herein, the human CII-3 gene present on MAC8.2.3 is an example of an endogenous, single copy gene useful as a cloning site. If desired, an exogenous nucleic acid sequence can be cloned into such a single copy nucleic acid sequence present on the MAC using, for example, methods of homologous recombination as disclosed herein. For example, where an exogenous nucleic acid sequence is cloned into the CII-3 gene present on MAC8.2.3, the exogenous nucleic acid sequence is linked to targeting sequences comprising a portion of the CII-3 gene.

As used herein, the term "portion of a nucleic acid sequence of a human CII-3 gene" means a nucleotide sequence of the human CII-3 gene that is of a sufficient length to allow specific hybridization of the sequence to an endogenous human CII-3 gene. Specific hybridization can be identified by performing routine hybridization reactions with a selected nucleotide sequence of the CII-3 under stringent hybridization conditions. Generally, such a nucleotide sequence is at least about 14 nucleotides in length. In addition, since specificity increases with increasing length of a sequence, a nucleotide sequence that is at least about 18 nucleotides in length can be particularly useful as a targeting sequence. Furthermore, it is well known that the efficiency of homologous recombination increases with the length of the targeting sequence. Thus, targeting sequences of at least about 100 nucleotides and up to several kilobases can provide relatively high efficiency of insertion of an exogenous nucleic acid sequence into a particular locus. Such targeting sequences can be selected, for example, from the genomic CII-3 sequences disclosed herein as SEQ ID NOS: 3, 5, 6 or 7. Such a sequence also can be selected from SEQ ID NO: 4, which is an exon of the CII-3 gene, or can be selected from nucleotide sequences of the CII-3 cDNA (SEQ ID NO: 1). Using methods as disclosed in Example II, additional sequences of the human CII-3 gene can be obtained, thereby providing substantially longer targeting sequences useful in the invention.

It should be recognized that when a targeting sequeace is selected from a coding region of a gene such as the human CII-3 gene, such a sequence generally must be longer than a sequence selected from a noncoding region of the gene. With regard to the human CII-3 gene present on MAC8.2.3, for example, the presence of related human CII-3 genes or pseudogenes (see Example II) and the high degree of sequence identity shared between CII-3 coding sequences of different species can result in insertion of the targeting vector into genomic DNA sequences other than the human CII-3 gene present on MAC8.2.3. Such nonspecific insertion of a targeting vector can be minimized, for example, by including noncoding sequences such as intron sequences in a targeting sequence comprising a coding sequence. However, even if insertion of a targeting vector occurs in a gene other than the human CII-3 gene present on MAC8.2.3, such nonspecific insertions can be identified using methods as disclosed herein or otherwise known in the art. For example, nonspecific insertion can be identified by a change, or lack thereof, in the ability of host cells containing the MAC to survive under selective conditions. In addition, nonspecific insertion can be identified, for example, by fluorescence in situ hybridization.

In particular, a unique cloning site can be a nucleic acid molecule encoding a selectable marker, which can be an exogenous or endogenous nucleic acid sequence. As used herein, the term "selectable marker" means a nucleic acid sequence or a protein or peptide expressed therefrom that confers upon a cell containing the marker the characteristic that the cell can be identified among a population of cells that do not contain the marker. Thus, a cell containing a MAC expressing a selectable marker displays a phenotype that the cell did not display prior to expression of the selectable marker. A selectable marker can be a positive marker, which allows direct identification of a cell containing the marker, or can be negative marker, which kills a cell, thereby allowing identification of the cell indirectly by its absence. For convenience, reference is made herein to a MAC containing a selectable marker, since a MAC containing such a unique cloning site is exemplified. It should be recognized, however, that a MAC of the invention is characterized, in part, by containing a unique cloning site and that a selectable marker is one embodiment of a unique cloning site.

A selectable marker can confer upon a cell expressing the marker the ability to survive in an environment that otherwise kills cells not expressing the marker. The CII-3 gene is an example of such a selectable marker, which also is an endogenous nucleic acid sequence in MAC-8.2.3. The CII-3 gene is an example of a selectable marker that corrects a genetic defect in a mutant cell, such that the cell attains a wild type phenotype. The use of such a selectable marker requires that a mutant cell type is available, such that a mutant cell containing a MAC can be identified by expression of the marker. A diseased cell such as a muscle cell expressing a mutant dystrophin gene in a muscular dystrophy patient is another example of a mutant cell. Thus, a normal dystrophin gene can be a selectable marker, which, when introduced using a MAC into the mutant muscle cell, results in the previously mutant muscle cells attaining a normal muscle cell phenotype.

A selectable marker can allow a cell to survive in the presence of a drug that otherwise would kill the cell. Such selectable markers, include, for example, positive selectable markers that confer resistance to neomycin (geneticin; G418), puromycin or hygromycin B. In comparison, a selectable marker such as the *Herpes simplex* virus thymidine kinase (HSV-tk) is useful for either positive selection, for example, in a cell that is deficient in thymidine kinase activity, or negative selection, whereby cells expressing HSV-tk are killed by exposure to ganeyclovir. Such selectable markers are useful because they confer an identifiable phenotype on an otherwise normal cell and, therefore, do not require the availability of a mutant cell. These and other selectable markers are well known in the art and commercially available (see, for example, Sambrook et al., *Molecular Cloning: A laboratory manual* (Cold Spring Harbor Laboratory Press 1989), which is incorporated herein by reference; see pages 16.9–16.15).

In addition, a selectable marker can be a product that allows a cell containing the MAC to be identified visually among a population of cells, some of which do not contain the selectable marker. Examples of such selectable markers include the green fluorescent protein (GFP), which can be visualized by its fluorescence; the luciferase gene, which, when exposed to its substrate luciferin, can be visualized by its luminescence; and β-galactosidase (β-gal), which, when contacted with its substrate, produces a characteristic color. Such selectable markers are well known in the art and the nucleic acid sequences encoding these markers are commercially available (see Sambrook et al., supra, 1989).

As disclosed herein, a MAC was prepared by isolating and characterizing a portion of a human chromosome containing a selectable marker. Specifically, a portion of human chromosome 1, also called minichromosome 1, containing the human CII-3 gene was selected in Chinese hamster cells that have a mutation in the CII-3 gene. Although the minichromosomes have been partially characterized (Carine et al., supra, 1986; Carine et al., supra, 1989; see, also, Solus et al., *Somat. Cell Mol. Genet.* 381–391 (1988), which is incorporated herein by reference), prior to the present disclosure that the defect in the host Chinese hamster cells is due to a mutation in the CII-3 gene and that the defect is complemented by a normal human CII-3 gene present on the minichromosome 1, the minichromosome was not considered useful as a MAC.

MAC-8.2.3 was selected in mutant Chinese hamster fibroblasts that are defective in succinate dehydrogenase (SDH) activity (Scheffler, *J. Cell. Physiol.* 83:219–230 (1974); Soderberg et al., *Cell* 10:697–702 (1977), each of which is incorporated herein by reference). The SDH-deficient hamster cells, designated CCL16-B9, are the host cells that contain MAC-8.2.3 and are deposited as ATCC Accession No. ATCC CRL 11992 (CCL16-B9 cells containing MAC-8.2.3 also are known as XEWS.2.3 cells). As a result of the SDH deficiency, CCL16-B9 cells require glucose in the growth medium and cannot grow in medium in which galactose is substituted for glucose.

Succinate dehydrogenase is part of complex II of the mitochondrial electron transport chain, linking the reactions of the tricarboxylic acid cycle to oxidative phosphorylation. This complex consists of four polypeptide subunits: a 70 kiloDalton (kDa) flavoprotein (FP), a 27 kDa iron-containing protein (IP), and two small integral membrane anchor proteins (CII-3 and CII-4; 15 and 7–9 kDa, respectively). Each subunit is encoded by a nuclear gene (SDH-1, SDH-2, SDH-3 and SDH-4, respectively) in eukaryotic cells. While an FP-IP complex, alone, can be dissociated from the inner mitochondrial membrane by chaotropic ions and assayed for succinate dehydrogenase activity using artificial electron acceptors, studies with yeast mutants indicate that the membrane anchor proteins are essential for the assembly of a functional complex II and SDH activity.

In order to identify the genetic defect in the CCL16-B9 hamster cells, somatic cell fusions were made between the mutant hamster cells and human cells and two independent primary (human x hamster) hybrids, XJM12.1.2 and XJM12.1.3, that grew in galactose-containing medium were obtained (Mascarello et al., supra, 1980). Secondary hybrids then were selected by fusing heavily irradiated XJM12.1.3 cells with the original mutant Chinese hamster CCL16-B9 cells and again selecting for cells that grew in galactose-containing medium (respiration competent hybrids; see Carine et al., supra, 1986).

Examination of the primary and secondary hybrid cell lines revealed the presence of a single human minichromosome consisting of a centromeric fragment of human chromosome 1 (Carine et al., supra, 1986; Carine et al., supra, 1989). Hybrid cells retaining an apparently intact human chromosome 1 also were examined. Spontaneous loss of the intact chromosome 1 resulted in loss of SDH activity and reversion to the respiration deficient condition; spontaneous segregation of the human minichromosome 1 has not been observed over many years in culture.

The minichromosomes first were observed in metaphase spreads in the light microscope after in situ hybridization with human Alu sequences. The minichromosome present in XEWS.2.3 also was examined by electron microscopy and was similar in length to the kinetochores. Based on the assumption that Alu sequences are uniformly distributed in the human genome, except in centromeres, telomeres and a few other regions containing tandem repeats, the minichromosome in 8.2.3 was estimated to contain about 1–2 million base pairs of human DNA, which is about 0.05% of the DNA contained in a normal haploid human genome (see Carine et al., supra, 1986; Carine et al., supra. 1989; see, also, Solus et al., supra, 1988).

All of the minichromosomes examined contained a small fraction of the pericentric chromatin from the long arm of human chromosome 1, as characterized by a satellite III DNA sequence present exclusively at 1q12. The minichromosomes also contained a-satellite DNA sequences, which are characteristic of human centromeres. Members of one such family were cloned from a genomic library prepared from the hybrid XJM12.1.3 and found to consist of a 340 bp Eco RI repeat containing two degenerate 170 bp monomers characteristic of alphoid DNA. In addition, another chromosome 1-specific α-satellite sequence, a 1.9 kb Hind III repeat, was present on the minichromosomes (Carine et al., supra, 1989).

A double labeling in situ hybridization experiment was performed using a-satellite and satellite III probes and visualized by electron microscopy. The results confirmed that the XEW8.2.3 minichromosome contains α-satellite sequences; satellite III DNA sequences were barely detectable. These results indicate that breakpoints occurred on either side of the centromere, retaining a small fraction of the pericentric heterochromatin on one side and about 1–2 million base pairs of the short arm of chromosome 1.

An anonymous single copy sequence was cloned from the minichromosome present in the XJM12.1.3 primary hybrid cell line. The single copy sequence also was present on the minichromosome present in the XJM12.2.2 primary hybrid cell line, as well as on the intact human chromosome 1. In comparison, the minichromosome present in the XEWS.2.3 secondary hybrid cell line, which was derived from XJM12.1.3, does not contain the anonymous sequence (Solus et al., supra, 1988), indicating that the anonymous sequence was located distal to the selectable CII-3 gene relative to the centromere (Carine et al., supra, 1989; Waye et al., *Genomics* 1:43–51 (1987); Willard, supra, 1987).

Indirect studies suggested that the mutant hamster cells had a defect in the gene for the IP subunit of SDH (Soderberg et al., supra, 1977). However, isolation and mapping of the genomic DNA, including the promoter, of the IP gene revealed that the IP gene was located at the distal end of the short arm of chromosome 1 (1p36.1-2) and was not present on the minichromosomes (Leckschat et al., *Somat. Cell Mol. Genet.* 19:505–511 (1993), which is incorporated herein by reference). Furthermore, an IP cDNA did not complement the respiration deficient condition when transfected into the mutant CCL16-B9 hamster cells. Thus, despite substantial characterization of the minichromosomes, a gene that complemented the SDH deficiency in the mutant hamster cells was not identified.

A bovine CII-3 cDNA (see Au et al., *Gene* 149:261–265 (1994), which is incorporated herein by reference) complements the SDH deficient phenotype in the mutant CCL16-B9 cells. In addition, a hamster CII-3 cDNA was isolated, the DNA sequence was determined and the encoded amino acid sequence was deduced. At the amino acid level there is about 82% identity between the wild type hamster and bovine CII-3 proteins. In comparison, the CII-3 cDNA isolated from mutant CCL16-B9 cells contains a single base mutation in the coding sequence for the CII-3 protein that produces a premature STOP codon and results in the truncation of 33 amino acids from the C-terminus and the SDH deficient phenotype in CCL16-B9 cells (see Example I). Furthermore, the gene encoding the human CII-3 subunit of complex II of the mitochondrial electron transport chain was mapped on human chromosome 1 and on the minichromosome, indicating that the human CII-3 gene is present within about 1–2 million base pairs of the centromere. These results indicated that MAC-8.2.3, which is present in the (human x hamster) secondary hybrid XEWS.2.3, cells, contains a normal copy of the human CII-3 gene that complements the mutation in the CCL16-B9 hamster cells.

As disclosed herein, a human CII-3 cDNA (SEQ ID NO: 1) has been cloned and expression of the human CII-3 cDNA also complements the mutation in the SDH-deficient B9 hamster cells. Furthermore, human genomic CII-3 DNA sequences were isolated (see FIG. 2; SEQ ID NOS: 3–7) and the human CII-3 gene was localized to MAC8.2.3, which is derived from human chromosome 1. The identification of this locus in MAC-8.2.3 provides a unique cloning site for inserting an exogenous nucleic acid sequence into MAC8.2.3.

Since MAC-8.2.3 contains a functional human CII-3 gene, a respiration-deficient hamster cell containing MAC-8.2.3 was identified by selecting cells that grew in galactose-containing medium. Thus, the CII-3 gene provides a selectable marker useful for identifying a CCL16-B9 hamster cell containing MAC-8.2.3. Significantly, identification of the CII-3 gene on MAC-8.2.3 provides a unique locus useful for site specific insertion of an exogenous nucleic acid sequence, thus making MAC-8.2.3 useful as a vector.

A MAC of the invention is useful as a vector for delivering an exogenous nucleic acid sequence into a cell and provides significant advantages over previously known vectors. For example, a MAC can contain an exogenous nucleic acid sequence having several thousand base pairs (kbp) up to several million base pairs. Thus, a MAC can contain an entire gene such as the 2300 kbp dystrophin gene, which is mutated in muscular dystrophy patients. In addition, a MAC

is stably maintained as a single entity in a cell. Thus, a MAC provides the additional advantage that a gene contained in and expressed from a MAC produces a unit dosage of an encoded gene product. Furthermore, a MAC is replicated along with the normal complement of chromosomes in a cell and, therefore, is passed to all of the daughter cells following a mitotic or meiotic division. Also, a MAC does not integrate into the genomic DNA in a cell but is maintained as an autonomous entity. Accordingly, introduction of an exogenous nucleic acid molecule contained in a MAC into a cell obviates any concern that the exogenous nucleic acid molecule may integrate into and disrupt the function of a normal gene in the cell.

The identification of the unique CII-3 gene sequence on MAC-8.2.3 provides a target site, into which an exogenous nucleic acid sequence can be inserted. A MAC containing an exogenous nucleic acid sequence can be transferred into a mammalian cell such as a mammalian stem cell, where the exogenous nucleic acid sequence can be expressed, if desired. Methods for introducing an exogenous nucleic acid sequence into a defined nucleic acid sequence such as one or more of the sequences shown as SEQ ID NOS: 3–7 are disclosed herein or otherwise known in the art. For example, an exogenous nucleic acid sequence can be targeted into the CII-3 gene using homologous recombination methods as have been used to produce gene knock-outs in embryonic stem cells in mice (see, for example, Gossen and Vijg, *Trends Genet,* 9:27–31 (1993); Frohman and Martin, *Cell* 56:145–147 (1989); Capecchi, *Science* 244:1288–1292 (1989); Westphal and Gruss, *Ann. Rev. Cell Biol.* 5:181–196 (1989); Zijlstra et al., *Nature* 342:435–438 (1989), each of which is incorporated herein by reference). In particular, the human CII-3 genomic DNA sequences shown as SEQ ID NOS: 3, 5, 6 or 7 can be useful in a targeting vector for homologous recombination because these sequences do not contain coding sequences or regulatory sequences and, therefore, are expected to occur as unique sequences in a cell that does not otherwise contain a human chromosome 1. A dominant selectable marker conferring, for example, neomycin resistance or puromycin resistance also can be introduced into MAC-8.2.3, thus facilitating selection and identification of virtually any mammalian cell containing the MAC (see, for example, Ayares et al., *Proc. Natl. Acad. Sci.. USA* 83:5199–5203 (1986), which is incorporated herein by reference).

A characteristic of a MAC that makes it particularly useful a vector is that an exogenous nucleic acid sequence can be inserted into a unique cloning site present in the MAC in a site specific manner. A particularly useful method of introducing an exogenous nucleic acid sequence into a MAC in a site specific manner utilizes a recombinase and recombinase recognition site, wherein the recombinase recognition site provides a unique cloning site. For example, site specific integration using the Cre recombinase and loxP recombinase recognition site from phage P1 (see Sauer, *Meth. Enzymol.* 225:890–900 (1993), which is incorporated herein by reference) or the yeast FLP/FRT system (see O'Gorman et al., *Science* 251:1351–1355 (1991), which is incorporated herein by reference) provides a convenient and efficient means for introducing an exogenous nucleic acid sequence into a MAC such as MAC-8.2.3 (see Example III and FIG. 1). Use of a site specific recombinase system for introducing a nucleic acid into a MAC provides the advantage that integration of the exogenous sequence does not disrupt or otherwise inactivate a characteristic of the MAC such as the ability to replicate or to segregate properly during cell division.

The FLP site specific recombinase has been characterized from *Saccharomyces cerevisiae* (Broach and Hicks, *Cell* 21:501–508 (1980), which is incorporated herein by reference) and the FLP recombination target (FRT) site has been identified (Jayaram, *Proc. Natl. Acad. Sci., USA* 82:5875–5879 (1985); Senecoff et al., *Proc. Natl. Acad. Sci., USA* 82:7270–7274 (1985), each of which is incorporated herein by reference). Functionality of the FRT/FLP system has been demonstrated in mammalian cells (O'Gorman et al., supra, 1991). In one experiment, a β-galactosidase (β-gal) gene sequence was disrupted by inserting a nucleic acid sequence flanked by two FRT sites; the insertion prevented expression of the β-gal gene product. Cotransfection into various mammalian cell lines of the disrupted β-gal gene and an FLP expression vector resulted in precise excision of the insert by the recombinase, leaving behind one FRT site, which preserved the β-gal reading frame, and restoring β-gal activity. In a second experiment, a single FRT site was inserted into a chromosome, then the cells were cotransfected with a vector containing a second FRT site and the FLP expression vector. The vector containing the FRT site was integrated specifically at the chromosome site containing the FRT site.

Site specific DNA recombination in mammalian cells also has been performed using the Cre recombinase of bacteriophage P1 and the loxP target site, which consists of 34 base pair repeats (see, for example, Orban et al., *Proc. Natl. Acad. Sci., USA* 89:6861–6865 (1992); Fukushige and Sauer, *Proc. Natl. Acad. Sci., USA* 89:7905–7909 (1992); Lakso et al., *Proc. Natl. Acad. Sci., USA* 89:6232–6236 (1992), each of which is incorporated herein by reference). For example, transgenic mice having a loxP-(β-gal)-loxP transgene positioned at a unique site have been produced (Orban et al., supra, 1992). When mated with transgenic mice carrying a Cre gene under control of the lck promoter, which is active only in thymocytes, doubly transgenic mice expressed the Cre recombinase, resulting in Cre-mediated recombination and excision of the β-gal gene in a cell specific manner. The loxP-Cre system also was used to delete a DNA polymerase b gene segment in T cells and to delete specific segments in the IgH locus (Gu et al., *Science* 265:103–106 (1994); Gu et al., *Cell* 73:1155–1164 (1993), each of which is incorporated herein by reference). In addition, purified Cre recombinase was introduced directly into osteosarcoma cells by lipofection and catalyzed site specific integration of a loxP targeting (Baubonis and Sauer, *Nucl. Acids Res.* 21:2025–2029 (1993), which is incorporated herein by reference).

In addition to its utility as a vector, a MAC such as MAC-8.2.3 can be used to identify the essential elements of a mammalian chromosome, including the nucleic acid sequences required to confer activity as a centromere, a telomere or an origin of DNA replication. Furthermore, large genomic DNA fragments cloned into a MAC provide a system for identifying and characterizing nucleic acid sequences required for coordinate regulation of gene complexes such as the immunoglobulin gene locus. Also, the ability of a MAC to segregate in a completely stable manner during mitosis provides a system for defining the mechanisms and factors involved in this process.

MAC-8.2.3, for example, contains a human chromosome 1 centromere that functions appropriately in Chinese hamster cell line (Carine et al., supra, 1989). human chromosomes characteristically have large tandem repeats of the alphoid family of satellite repeats at their centromere; individual chromosomes can be distinguished by which member (s) of the alphoid family of repeats is present, based on

restriction mapping and high stringency hybridizations (see, for example, Waye and Willard, *Nucl. Acids Res.* 15:7549–7569 (1987); Willard et al., *Trends Genet.* 3:192–198 (1990)). Since the human centromere alphoid sequences do not cross-hybridize with Chinese hamster DNA sequences, the centromere sequences of hamster and human chromosomes are substantially different. Nevertheless, human chromosomes, including MAC-8.2.3, are stably maintained in (human x hamster) hybrid cell lines. The use of MAC-8.2.3 provides a unique system for identifying the mechanisms involved in maintaining chromosome stability in a cell.

An essential feature of centromeric DNA sequences is the ability to become associated with special proteins to form a unique type of chromatin to which the proteins of the kinetochore become attached. Only a few such proteins have been identified. Kinetochores likely consist of a series of repeated structural motifs because more than 10 microtubules attach to each side of a metaphase chromosome. A MAC such as MAC-8.2.3 is useful for identifying the proteins involved in kinetochore formation and spindle fiber attachment. An understanding of the factors involved in spindle fiber attachment to a centromere can provide insight into the mechanism responsible for appropriate chromosome segregation during mitosis. Such an understanding can lead to the development of methods for preventing, for example, improper segregation, which can result in trisomy or in loss of a chromosome in a daughter cell.

The present invention also provides methods for preparing a MAC, comprising fragmenting a parental chromosome and selecting a centromeric fragment of the chromosome containing less than about 0.1% of the DNA present in a normal haploid mammalian genome containing the parental chromosome. The MAC is selected based on the presence of a selectable marker on the centromeric fragment, which further provides a unique cloning site that can be used as a site to insert an exogenous nucleic acid sequence or that can be further modified, for example, to contain a recombinase recognition site.

As used herein, the term "parental chromosome" means the normal cellular chromosome from which the MAC was derived. For example, MAC-8.2.3 was derived from and contains the centromere of human chromosome 1, which, therefore, was the parental chromosome of MAC-8.2.3 (see Example I). A MAC is prepared by obtaining a centromeric fragment of a chromosome containing a selectable marker. If desired, the MAC can be genetically engineered to provide one or more desirable characteristics. In particular, a MAC can be genetically engineered to contain, in addition to the selectable marker, an exogenous nucleic acid sequence such as a gene or a cDNA, which can encode a second selectable marker; an entire genetic locus, including regulatory elements such as enhancers, which can be several kilobases upstream or downstream of a gene; or a randomly produced fragment of genomic DNA.

Yeast artificial chromosomes (YACs) have been developed by assembling essential elements of yeast DNA, including centromeres, telomeres and replication origins (Burke et al., *Science* 236:806–812 (1987); Schlessinger, *Trends Genet.* 6:248–258 (1990)). However, it is not possible to apply the methods used in constructing a YAC similarly to construct a MAC because the essential elements such as mammalian origins of DNA replication and mammalian centromeres are not well characterized (see Huxley et al., *BioTechnology* 12:586–590 (1994); Brown, *Curr. Opin. Genet. Devel.* 2:479–486 (1992); Lewin, *J. NIH Res.* 7:42–46 (1995)).

As disclosed herein, a MAC can be prepared, for example, by telomere associated chromosome truncation or by irradiating a cell at a dose that causes fragmentation of the chromosomes in the cell and selecting therefrom a MAC based on the presence of an endogenous selectable marker located near the centromere (pericentric). Such pericentric endogenous selectable markers include, for example, the CII-3 gene or another gene that can be identified, for example, by searching in the Human Genome Database (GDB; v.6.0) accessible via the Internet at http://gdbwww.gdb.org/, which is incorporated herein by reference.

Since pericentric endogenous selective markers are expected to be rare in mammalian chromosome, a selectable marker generally will be randomly or site specifically inserted into the pericentric region of a chromosome. For example, a selectable marker conferring neomycin resistance can be inserted site specifically by homologous recombination into a gene that is located in a pericentric region of a chromosome. A pericentric gene can be identified in the GDB database (see above). For example, the neogene can be targeted into the gene encoding the high molecular weight neurofilament peptide, NF-H, which is a pericentric gene located on chromosome 1 at 1p12 (Lieberburg et al., *Proc. Natl. Acad. Sci., USA* 86:2463–2467 (1989), which is incorporated herein by reference), into the gene encoding an Fc Gamma receptor, which has been mapped to 1p12 (Mascarena et al., *Cytogenet. Cell Genet.* 73:157–163 (1996), which is incorporated herein by reference), or into any other pericentric gene. Following integration of the selectable marker, the chromosomes can be fragmented, for example, by telomere associated truncation, and a MAC can be obtained by somatic cell fusion, followed by selection of neomycin resistant hybrid cells and identification of a selected hybrid cell containing a MAC, as defined herein.

A selectable marker also can be targeted to an endogenous pericentric nucleic acid sequence other than a pericentric gene. For example, a selectable marker can be targeted using homologous recombination to a unique pericentric nucleic acid sequence or to a satellite DNA sequence, which generally is present in the region of the centromere (see Cariac et al., supra, 1989). Following integration of the selectable marker into the chromosome, a MAC is obtained, for example, by fragmenting the chromosomes containing the selectable marker, fusing the cells containing the fragmented chromosomes with a second cell line, which can be the same cell type as the first cell line, and selecting hybrid cells that contain a centromeric fragment of a chromosome containing the selectable marker, wherein the centromeric fragment has the characteristics of a MAC. Thus, based on the methods disclosed herein, the skilled artisan can prepare a MAC having characteristics similar to MAC-8.2.3 or a MAC having other characteristics as desired.

The invention also provides methods of stably expressing a selectable marker in a cell, comprising introducing a MAC containing the selectable marker into the cell. For example, the human CII-3 gene product is a selectable marker that is stably expressed in mutant hamster CCL16-B9 cells, which do not express a functional hamster CII-3 gene product.

As used herein, the term "stably expressed" when used in reference to a selectable marker means that the nucleic acid molecule encoding the marker is maintained and expressed in a cell line. In particular, a selectable marker is stably expressed from generation to generation in a cell type that traverses the cell cycle and, ultimately, divides. The ability to stably express a selectable marker in a cell is due to the ability of a MAC to be replicated during the cell cycle and

to segregate with a daughter cell during cell division. It is recognized, however, that some cells such as muscle cells generally do not divide. Nevertheless, a selectable marker is considered to be stably expressed in a non-dividing cell if the MAC containing the selectable marker is stably and autonomously maintained in the cell and if the selectable marker is expressed as appropriate. In this regard, it is further recognized that the term "stably" when used in reference to the expression of a selectable marker does not necessarily mean that the marker is "always" or "constantly" expressed because expression of a selectable marker is regulated, in part, by the particular gene regulatory elements linked to the marker. For example, a selectable marker containing a promoter that is active only during a particular stage of the cell cycle or that is induced only when activated by a particular regulatory factor, nevertheless is considered stably expressed if the selectable marker is expressed at the appropriate time. Thus, a "stably expressed" marker is stably expressed with reference to the particular regulatory elements linked to the marker.

As disclosed herein, a MAC was obtained following irradiation of cells. A MAC also can be obtained using telomere associated chromosome truncation, which is based on the knowledge that a telomere defines the end of a chromosome (Farr et al., *EMBO J.* 14:5444–5454 (1995); Heller et al., *Proc. Natl. Acad. Sci., USA* 93:7125–2130 (1996); Brown et al., *Human Mol. Genet.* 3:1227–1230 (1994); Willard, *Proc. Natl. Acad. Sci., USA* 93:6847–6850 (1996), each of which is incorporated herein by reference). Essentially, a telomeric sequence consisting of tandem repeats of the sequence TTAGGG is inserted into chromosomal DNA. Depending on the number of telomere sequences inserted into a particular chromosome, a truncated chromosome, which contains a centromere, and one or more fragments of the chromosomal arms distal to the most centromeric insertion site are produced. Chromosomal fragments lacking centromeres ultimately are lost from the cells, whereas the truncated chromosome can be stably maintained, generally under selective pressure in a host cell.

Insertion of a telomere sequence into a chromosome can be targeted to a specific locus or can be random (see Examples III and IV). Specific targeting can be accomplished, for example, by homologous recombination into a known gene or other unique nucleic acid sequence present in the chromosome. Random insertion of telomere sequences can be accomplished using, for example, a vector containing a telomere sequence, including a linear vector containing the telomere sequence at one end.

Telomere associated chromosome truncation can be particularly useful for producing a MAC where pericentric genes or other unique pericentric nucleic acid sequences are targeted. For example, homologous recombination can be used to target a telomeric sequence to a pericentric gene such as the CII-3 gene (see Example IV). Where such targeting results in retention of the CII-3 gene in the truncated chromosome, a host cell containing the truncated chromosome can be selected based on the ability of SDH-deficient mutant cells containing the truncated chromosome to survive under the appropriate selection conditions (see Example I). It should be recognized, however, that targeted telomere associated chromosome truncation, when performed on a normal, full size chromosome, results in truncation only of the distal region of the arm containing the target site; the remaining chromosome arm generally is not affected. Thus, where site specific targeting of telomeres is used, a telomere must be introduced into a pericentric site on each arm of a selected chromosome in order to produce a useful MAC.

If desired, a telomere sequence can be linked to a nucleic acid sequence encoding a selectable marker. For example, where specific targeting of the sequence is accomplished by homologous recombination, a targeting vector comprising the telomere sequence, the sequences homologous to the target site, and the nucleic acid sequence encoding the selectable marker is introduced in the cell containing the appropriate chromosome. Following insertion of the targeting vector into the chromosome, cells containing the vector can be selected under the appropriate conditions and truncated chromosomes can be identified using routine cytogenetic methods. The inclusion of a selectable marker with the telomere sequence in a targeting vector can be particularly useful where the targeted gene such as the CII-3 gene, which otherwise is a selectable marker, is lost due to the truncation event or where the targeted pericentric sequence does not provide a means of selection or provides an inconvenient means of selection.

As disclosed herein, telomere associated truncation of a minichromosome such as a minichromosome maintained in the hybrid XJM12.1.2 and XJM12.1.3 cells (Mascarello et al., supra, 1980) or of a MAC such as MAC8.2.3 can provide significant advantages over methods of chromosome truncation previously described. For example, previous truncation methods have started with normal, full size chromosomes and have required several rounds of truncation and selection in order to obtain a minichromosome approaching a size useful as a MAC (see, for example, Heller et al., supra, 1996). However, the minichromosomes obtained following such sequential truncations contained rearrangements (see Willard, supra, 1996), which raises a question as to whether the minichromosome can function as a stably maintained entity, particularly over a long period of time. The occurrence of such rearrangements also can complicate specific targeting into the minichromosome by changing the locus, orientation or contiguity of the target site. Furthermore, even if the target site, itself, remains unaffected by a rearrangement, an unexpected level of expression of an introduced nucleic acid sequence can result due, for example, to the loss of a regulatory sequence normally associated with the target site or to the gain of a regulatory element due to the rearrangement.

In comparison to performing telomere associated chromosome truncation with normal, full size chromosomes, a stably maintained minichromosome such as the minichromosome present in XJM12.1.2 cells or in XJM12.1.3 cells provides a smaller initial target for telomere associated truncation. Thus, a truncated minichromosome useful as a MAC can be obtained after a single round of truncation and selection, limiting the probability that undesirable rearrangement of the truncated minichromosome will occur.

Similarly, a single round of telomere associated truncation can be used on a MAC such as MAC8.2.3 in order to obtain a smaller MAC, which can facilitate manipulation and transfer of the MAC (see Example IV). Specifically, a telomere sequence can be introduced into the CII-3 gene present on MAC8.2.3 by using a unique target sequence such as a sequence shown as SEQ ID NOS: 3, 5, 6 or 7 as the targeting sequence for homologous recombination. Alternatively, a telomere sequence can be introduced proximal or distal to the CII-3 gene with respect to the centromere, depending upon whether it is desired to maintain a functional CII-3 gene in the truncated MAC. As an additional advantage, targeting of a telomere sequence to the 5'-end or the 3'-end of the CII-3 gene provides a convenient means for determining the orientation and the relative position of the gene on MAC8.2.3.

A MAC can be transferred from a host cell into a second cell. For convenience, a host cell containing a MAC to be transferred is referred to herein as a "donor" cell, whereas the cell into which the MAC is transferred is referred to as a "recipient" cell. Various methods are known for transferring a MAC, which is a centromeric fragment of a chromosome, into a recipient cell. For example, a MAC can be transferred from a donor cell to a recipient cell by somatic cell fusion (see, for example, Carine et al., supra, 1986). Hybrid cells containing the MAC can be identified based on expression of the selectable marker present in the MAC. Although one or more donor cell chromosomes also will be transferred into the hybrid cells, such donor cell chromosomes generally are lost during passage of the cells because there is no selective pressure for maintaining the donor cell chromosomes in the hybrid cell. Examination of clones of hybrid cells can be used to identify hybrids containing only the MAC from the donor cells.

A host donor cell also can be treated with a mitotic spindle inhibitor such as colchicine, which results in the formation of micronuclei, then with cytochalasin B, which results in the extrusion of microcells, which contain one or a few chromosomes, including the MAC, and which can be fused to recipient cells (see, for example, Ege and Ringertz, *Expt. Cell Res.* 87:378–392 (1974); Fournier and Ruddle, *Proc. Natl. Acad. Sci., USA* 74:319–323 (1977), each of which is incorporated herein by reference. Fusion of recipient cells with microcells greatly reduces the transfer of donor cell chromosomes to recipient cells. In addition, minichromosomes can be isolated by fluorescence activated cell sorting (FACS; see Ferguson-Smith, in *Molecular Biology and Biotechnology: A comprehensive desk reference* (ed. Meyers; VCH Publ., NY; 1995) pages 354–359; Krumlauf et al., *Proc. Natl. Acad. Sci., USA* 79:2971–2975 (1982); Wallace et al., *Nucl. Acids Res.* 17:1665–1678 (1989), each of which is incorporated herein by reference). Since a MAC is much smaller than the smallest intact chromosome, isolation of MACs using FACS provides a means to obtain substantially purified MACs, which can be introduced into a recipient cell, for example, by microinjection.

A MAC containing a selectable marker is useful for stably expressing the selectable marker in a cell. A MAC containing a neo gene and a gene or cDNA encoding gene product of interest can be transferred into a diseased cell, wherein expression of the gene product complements the genetic defect and results in the cell attaining a normal phenotype. In general, a diseased cell is obtained from a patient, the MAC is transferred from a host cell into the recipient diseased cell in vitro, then the recipient cell containing the MAC is reintroduced back into the patient. Thus, a MAC can be useful as a vector for gene therapy.

In particular, a MAC is useful for introducing a large gene such as the dystrophin gene into a recipient cell because other mammalian cell vectors cannot contain such a large gene. However, a MAC is useful for introducing any gene or cDNA into a cell. Furthermore, the use of a MAC for gene therapy provides the advantage that the gene product of interest is produced in a unit dosage, since generally only a single MAC will be present in the recipient cell. In addition, a MAC is stably and autonomously maintained in each daughter cell following division of the parental cell. Thus, a MAC can be particularly useful for introducing an exogenous nucleic acid molecule into a stem cell such as a bone marrow stem cell because all the cells propagated from the stem cell will stably express the exogenous nucleic acid.

It is further recognized that a MAC is particularly useful for expressing a mammalian gene product in vitro. A MAC containing an exogenous nucleic sequence can be introduced into a mammalian cell for the purpose of expressing and collecting a mammalian gene product encoded by the nucleic acid. Mammalian cells containing a MAC can be grown in large quantities in vitro in a bioreactor under conditions that allow expression of an exogenous nucleic acid sequence contained in the MAC.

Use of a MAC to express a mammalian gene product in a mammalian cell provides significant advantages over methods for expressing the gene product in other cell types such as insect cells or bacterial cells because appropriate post-translational modifications such as glycosylation or phosphorylation of the expressed gene product can occur in a mammalian host cell. In addition, use of a MAC to introduce an exogenous nucleic acid sequence into a mammalian cell provides significant advantages over the use of other vectors. For example, the MAC is stably expressed in the mammalian cells and, therefore, is passed from generation to generation in dividing cells. In addition, a MAC is maintained as an autonomous entity in a cell and, therefore, does not integrate into the genomic DNA, where it can disrupt the regulation or expression of endogenous gene products. Thus, the present invention provides a method of producing an exogenous mammalian gene product in a cell by introducing a MAC containing an exogenous nucleic acid sequence encoding a mammalian gene product into a mammalian cell and expressing the gene product. Such a method allows in vitro production of large amounts of essentially any mammalian protein, provided the nucleic acid sequence encoding the protein is known.

A MAC also is useful for producing a transgenic mammal such as a mouse, cow, goat or sheep expressing a gene of interest (see Example V). A MAC is particularly useful for this purpose because the MAC is stably and autonomously maintained in all of the cells containing the MAC. However, it is not necessary that the exogenous nucleic acid sequence be expressed in every cell containing the MAC because expression of the exogenous nucleic acid sequence is dependent on the particular characteristics of the promoter that directs its expression. Thus, the introduction of the MAC into an embryonic stem cell or into an ovum provides a means to produce a transgenic mammal having a desirable characteristic. For example, a MAC containing a selectable marker can be microinjected into an ovum, which can fertilized at the time of microinjection or can be unfertilized, then fertilized following microinjection. The MAC-containing zygote then is implanted into a pregnant or pseudopregnant female and the newborn mammals are examined for expression of the selectable marker. Transgenic mammals expressing the selectable marker are thereby produced.

The following examples are intended to illustrate but not limit the present invention.

EXAMPLE I

Preparation and Characterization of a Mammalian Artificial Chromosome

This example provides methods for preparing and characterizing MAC-8.2.3, which is contained in XEWS.2.3 cells deposited on Oct. 31, 1995, as ATCC Accession No. ATCC CRL 11992.

The various cell lines used in this study and the hybrids derived from the fusion of the Chinese hamster mutant cells with human cells have been described previously (Mascarello, supra, 1980; Carine, supra, 1986, 1989).

Briefly, CLL16-B1 is a Chinese hamster lung fibroblast from which the SDH-deficient mutant cell line CLL16-B9 was selected (Ditta et al., *Somat. Cell Genet.* 2:331–344 (1976). which is incorporated herein by reference; Soderberg et al., supra, 1977). The hybrid cell lines XJM5.1.1(+) and XJM12.1.3 were derived from the fusion of the CCL16-B9 cells with human lymphocytes or HT1080 fibrosarcoma cells (Croce, *Proc. Natl. Acad. Sci., USA* 73:3248–3252 (1976), which is incorporated herein by reference) and by selection of respiration-competent cells. The hybrid cell line XJM5.1.1(–) was a respiration-deficient segregant which had lost human chromosome 1. XJM12.1.3 was one of two independent hybrids with a human minichromosome. The secondary hybrids XEWS.2.3 and XEW9.10.4 were isolated after fusing irradiated XJM12.1.3 hybrids with CCL16-B9 cells and selecting for SDH-positive hybrids (Carine et al., supra, 1986).

All cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 5 mM glucose and 10% fetal calf serum. The same medium with glucose substituted by galactose (DME-GAL) was used to select for or maintain respiration-competent cells or hybrids (Ditta et al., supra, 1976; Scheffler et al., in *Biomedical and Clinical Aspects of Coenzyme Q*, pages 245–253 (Folders and Yamamura, eds.; Elsevier/North Holland Biomed. Press, Amsterdam; 1981), which is incorporated herein by reference).

The partial cDNA encoding the bovine heart CII-3 subunit of complex II (Yu et al., *J. Biol. Chem.* 267:24508–24515 (1992), which is incorporated herein by reference) and the complete bovine cDNA (Cochran et al., *Biochem. Biophys. Acta* 1188:162–166 (1994), which is incorporated herein by reference) were obtained. Southern blot analysis was performed on human, hamster and hybrid genomic DNA using the bovine CII-3 cDNA probe. Southern blot and northern blot analyses were performed using standard methods (Sambrook et al., supra, 1989); probes were labeled by the random primer method. Restriction enzymes were obtained from New England Biolabs (Beverly, Mass.) and used according to the manufacturer's instructions. (α-^{32}P)-dCTP was from ICN Pharmaceuticals (Irvine, Calif.).

Southern blot analysis revealed that the (human x hamster) hybrid cells contained, in addition to the hamster chromosomes, a small number of human chromosomes. The hybrid XJM5.1.1.(+) contains an intact human chromosome 1, while the hybrid XJM5.1.1.(–) has lost the entire chromosome 1 during subsequent culture in nonselective conditions (Mascarello et al., supra, 1980). The hybrids XJM12.1.3 and XJM12.2.2 contain a human minichromosome, with a few million base pairs of DNA from the short arm of chromosome 1; the secondary hybrids XEW8.2.3 and XEW9.10.4 were derived from XJM12.1.3 after irradiation and contain a human minichromosome with 1–2 million base pairs of DNA from the short arm.

The bovine cDNA probe hybridized with hamster and with human restriction fragments even at relatively high stringency. Multiple bands were present, particularly in DNA samples obtained from the human cells. This result indicates that the human CII-3 gene consists of exons and introns or that multiple CII-3 genes or pseudogenes are present in the human genome (see Example II). Several different restriction enzymes were used in these investigations, including some that do not cleave the bovine cDNA (Xba I, Eco RI, Pst I). Interestingly, the samples from the human cells contain bands that are not present in DNA obtained from hybrids containing a human minichromosome. However, some bands are shared between total human DNA and the minichromosome present in the hybrid cells. As expected, all of the hybrid cell lines contain the band characteristic of hamster genomic DNA. These results indicate that the gene for the CII-3 subunit is found on the human minichromosome, including the minichromosome present in XEW8.2.3 cells.

In order to confirm that the genetic defect in the mutant hamster cells is due to an aberrant CII-3 gene or gene product, the ability of the bovine CII-3 cDNA to complement the SDH deficient condition of CCL16-B9 cells was examined. The complete bovine cDNA was excised from its pUCI18 vector as an Eco RI fragment and cloned into the mammalian expression vector pcDNA3 (Invitrogen; San Diego, Calif.) for the complementation analysis. Cells were grown to 50% confluency and transfected with the pcDNA3-CII-3 construct. As one control, pcDNA3 containing an unrelated cDNA insert was used and in a second control, no vector was added to the transfection mixture.

Transfection was performed using the "LIPOFECTAMINE" reagent (GIBCO BRL; Grand Island, Mich.); conditions for optimal transfection efficiency of the CCL16-B9 cells were established using the eukaryotic assay vector pCH110 containing the β-gal gene (Pharmacia; Piscataway, N.J.). Selective medium, either DMEM containing 800 μg/ml G418, which selects for expression of the neo gene, or DMEM-galactose, which selects for respiration competent cells, was added 2 days or 4 days after transfection. After 8 days some, plates selected with G418 were switched to DMEM-galactose. Stable transfectants were maintained in DMEM-galactose containing 400 μg/ml G418.

Complementation of the defective mitochondrial function is not instantaneous because new functional complexes must be assembled in the mitochondria and time is required to accumulate levels of complex II that are adequate to support respiration and oxidative phosphorylation (Mascarello et al., supra, 1980; Carine et al., supra, 1989). Similarly, a lag period was observed in the transfected cultures when direct selection began within a few days after transfection. However, after about two weeks, cells began to divide in DMEM-galactose. In contrast, cultures that were not transfected with any vector or were transfected with the vector containing an unrelated cDNA and the neogene, no proliferation was observed after the switch to the DMEM-galactose medium and, after a few days, the cells died and became dislodged from the plate. These results demonstrate that the bovine CII-3 cDNA complements the SDH deficiency in CCL16-B9 cells.

The ability of the bovine CII-3 cDNA to restore SDH activity in the mutant CCL16-B9 cells also was examined. SDH activity was determined using the assay of Green and Narahara (*J. Histochem. Cytochem.* 28:408–412 (1980). which is incorporated herein by reference), which measures the succinate-dependent reduction of the analogue 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride. The reaction product was quantitated spectrophotometrically after extraction with ethanol. Mitochondria were isolated by differential centrifugation as previously described (Ditta et al., supra, 1976; Soderberg et al., supra, 1977).

The activity measured in mitochondria from wild type hamster cells was inhibited almost completely by malonate, which indicates the assay is specific for SDH. Mitochondria from CCL16-B9 cells had less than 5% of the activity of from CCL16-B9 cells had less than 5% of the activity of wild type mitochondria. In the complemented mutants, the activity was restored to levels ranging from 30 to 50% of wild type.

These results indicate that the SDH deficiency observed in CCL16-B9 cells is due to a defect in either the CII-3 gene or the CII-3 gene product. In order to determine whether the defect was due to a mutation in the hamster CII-3 gene, hamster CII-3 cDNA was cloned. The availability of the bovine cDNA sequence allowed the design of primers for cloning the hamster CII-3 cDNA from wild type and mutant cells using a polymerase chain reaction (PCR). Computer analysis of the bovine CII-3 cDNA sequence was performed in order to avoid regions of the sequence likely to form hairpins or to dimerize. Sequences within the coding sequence were selected because the untranslated regions of the bovine and hamster sequences are more likely to have diverged.

Based on the computer search, two oligonucleotide sequences were prepared: 5'-TGCCAGCCCTACAGAGGACAACAC-3' (SEQ ID NO: 15) and 5'-CTGGAGTAAGAACACTACTTTAAACCGTCC-3' (SEQ ID NO: 16). Eventually only use of the primer corresponding to the 3'-end of the coding sequence (SEQ ID NO: 15) was successful for cloning a large portion of the CII-3 cDNA from wild type and mutant hamster cells by the 5' RACE protocol (Frohman et al., *Proc, Natl. Acad. Sci., USA* 85:8998–9002 (1988), which is incorporated herein by reference; see Example II).

Reverse transcription-PCR (RT-PCR) did not yield a product when the two specific primers were used. Thus, the 5' and 3' RACE methods were attempted. Although the 3' RACE protocol was not successful, the 5' RACE protocol resulted in isolation of PCR products that included the 5' untranslated region of hamster CII-3 mRNA and all but 9 nucleotides at the 3' end of the open reading frame. Four independent RT-PCR reactions were carried out with each of the wild type and mutant RNAs as templates. The PCR products were cloned into the pGEM-T vector (Promega; Madison, Wis.) for direct sequencing. DNA sequencing was performed using the Sequenase 2.0 kit (United States Biochemical; Cleveland, Ohio) and the SP6 and T7 sequencing primers, as described by manufacturer.

The nucleotide sequence of the wild type hamster CII-3 cDNA is accessible from the GenBank/EMBL Data Bank at Accession No. U81241, which is incorporated herein by reference. All of the clones obtained from mutant cells contained a G→A transition in codon 137, resulting in the conversion of a tryptophan codon to a STOP codon. As a result of the mutation in the CII-3 cDNA, 33 amino acids normally found at the C-terminus of the translated protein are absent. A comparison of the bovine and hamster CII-3 cDNA sequences demonstrated 86% conservation at the nucleotide level and 82% conservation at the amino acid sequence level. The changes are largely conservative changes and are scattered throughout the entire peptide.

The results of these studies demonstrate that the defect in the SDH deficient CCL16-B9 hamster cell line is due to a genetic mutation at a single nucleotide in the CII-3 gene, resulting in production of a truncated CII-3 protein. In addition, the results demonstrate that the corresponding human CII-3 gene is very closely linked to the human chromosome 1 centromere. Based on these results, a unique DNA sequence has been defined on the minichromosome that is present in XEWS.2.3 cells; the minichromosome is designated herein as MAC-8.2.3.

EXAMPLE II

CII-3 cDNA and Partial Genomic DNA Sequence

This example describes methods for isolating nucleic acid sequences encoding the CII-3 subunit of complex II of the mitochondrial electron transport system.

A. Human CII-3 cDNA:

A complete human cDNA encoding CII-3 was cloned from a HeLa cell cDNA library using the 5' and 3' RACE method and sequenced by the dideoxy chain termination method. The first strand cDNA library was produced by reverse transcription of 1 μg total RNA from HeLa cells using the dT17 anchor primer 5'-GACTCGAGTCGACATCGATTTTTTTTTTTTT-TTTT-3' (SEQ ID NO: 12) and "SUPERSCRIPT II" (RNAse H(–); BRL; Gaithersburg, Md.) at 48° C., followed by poly C tailing using terminal deoxynucleotidyl transferase (BRL).

The 5'-cDNA was cloned by PCR amplification of the first strand cDNA library using a gene specific primer, 5'-GCCAGCCCCATAGAGGACAACAC-3' (SEQ ID NO: 13) and the dG15 anchor primer, 5'-GACTAGTCGACTGCAGGGGGGGGGGGGGG-3' (SEQ ID NO: 14). The 550 base pair (bp) PCR product was cloned directly into the pGEM-T vector (Promega). The 3'-cDNA was cloned by PCR amplification of the first strand cDNA library using the gene specific primer 5'-GACTCGAGTCGACATCGATTTTTTTTTTTTT-TTTT-3' (SEQ ID NO: 12). The 1000 bp PCR product was cloned directly into pGEM-T.

The CII-3 cDNA contains a 27 nucleotide 5'-untranslated sequence, a 510 nucleotide coding sequence, and a 779 nucleotide 3'-untranslated sequence (SEQ ID NO: 1; see, also, GenBank Accession No. U57877). The human CII-3 amino acid sequence as deduced from SEQ ID NO: 1 is shown as SEQ ID NO: 2. Three independent clones were isolated and sequenced from the cDNA library and each contained the same CII-3 cDNA sequence. This result suggests that only a single CII-3 gene is expressed in human cells. Northern blot analysis also identified only a single band, although it is unknown whether the band corresponded to one or more RNA transcripts. Significantly, expression the cloned human CII-3 cDNA (SEQ ID NO: 1) in the SDH-deficient CCL16-B9 cells complemented the mutation, thereby allowing the cells to survive under the selective conditions (see Example I). This result confirms that the cloned human cDNA sequence encodes a functional CII-3 gene product.

B. Human CII-3 Genomic DNA Sequencing:

Human genomic DNA cloned in the lambda DASH vector was purchased from Stratagene (La Jolla, Calif.) and screened using a bovine CII-3 cDNA (Yu et al., supra, 1992; Cochran et al., supra, 1994; see, also, Ostveen et al., *J. Biol. Chem.* 270:26104–26108 (1995), which is incorporated herein by reference). Four genomic clones containing CII-3 DNA sequences were isolated and partially characterized. One clone (JS18) contained the complete CII-3 coding sequence, with no evidence of introns. However, the coding sequence contained two in-frame stop codons and, therefore, likely represents a pseudogene. A second clone (JS5.1) also contained the complete CII-3 coding sequence, as well as sequences characteristic of a 5'- and 3'-untranslated sequence, but no introns. However, no stop codon was present in this coding sequence. Thus, it is unclear whether this sequence is expressed or is a pseudogene, although, based on the cDNA cloning, it appears that only a single CII-3 gene product is expressed.

The two remaining clones (JS2 and JS5.2) contained overlapping sequences as determined by restriction mapping. The genomic sequence in JS2 was digested with Not I and Eco RI and a 1.8 kbp NotI/EcoRI fragment was subcloned into a "BLUESCRIPT" vector (Stratagene) to

produce. pJOS2 (see FIG. 2). Partial DNA sequencing of pJOS2 confirmed that the subcloned genomic sequence contained a sequence (SEQ ID NO: 4; see FIG. 2. "Exon-A") that is identical to a portion of the cloned human CII-3 cDNA (SEQ ID NO: 1).

A portion of the subcloned genomic sequence is disclosed herein as Intron-A (SEQ ID NO: 3; 257 bp), Exon-A (SEQ ID NO: 4; 164 bp) and Intron-B (SEQ ID NO: 5; 173 bp; see, also, FIG. 2). Exon-A (SEQ ID NO: 4), which corresponds to nucleotides 268 to 431 of the human CII-3 cDNA (SEQ ID NO: 1), is bordered on its 5'-end by Intron-A (SEQ ID NO: 3) and on its 3'-end by Intron-B (SEQ ID NO: 5). Additional sequences bordering the 5-end (SEQ ID NO: 6; 261 bp) and 3'-end (SEQ ID NO: 7; 333 bp) of the 1.8 kbp genomic DNA fragment in pJOS2 also have been determined (FIG. 2; "X" and "Y," respectively). The exon and introns are referred to by letters because the complete structure of the human CII-3 gene has not yet been determined. The sequences shown as SEQ ID NOS: 5 and 6 are referred to as "X" and "Y," respectively, since it is not clear whether they constitute intron or exon sequences or portions of both. Reference to the "5'-end" and "3'-end" indicate the position relative to the reading frame encoded by Exon-A, based on its identity to the human CII-3 cDNA (SEQ ID NO: 1).

DNA sequencing was performed using the following primers: pJOS2.Rev, 5'-TGGTGAAACCCTGTCTCTAC-3' (SEQ ID NO: 8); pJOS2.T7, 5'-TCTATGCCTTCAGGGATCTC-3'-(SEQ ID NO: 9); BuQPS1.Forw1, 5'-ACTTGTGAAGTCCCTGTGTC (SEQ ID NO: 10); and HuQPS1.Rev3, 5'-AAGTGTCGGATCCCATTCCA-3' (SEQ ID NO: 11). The pJOS2.Rev and pJOS2.T7 primers were prepared based on sequences of the genomic subclone that were obtained using the "universal" T7 and reverse primers specific for the cloning vector. The BuQPS1.Forw1 and HuQPS1.Rev3 primers were designed based on the human CII-3 cDNA sequence (SEQ ID NO: 1).

The HuQPS1.Forw1 and pJOS2.T7 primers, which are complementary to sequences of Exon-A and "Y," respectively (see FIG. 2), also were used to amplify genomic DNA obtained from human HeLa cells; hamster B9 cells; XJM5.1.1(+) cells, which are hamster cells containing a complete human chromosome 1 (see Example I); XJM5.1.1 (−) cells, which are derived from XJM5.1.1(+) cells that have lost the human chromosome 1; or XEWS.2.3 cells, which are hamster cells containing MAC8.2.3. The amplification products were separated by polyacrylamide gel electrophoresis and visualized by ethidium bromide staining and ultraviolet irradiation. A band migrating at about 1.05 kbp, which is the expected size of the amplified portion of the human CII-3 gene, was obtained from genomic DNA obtained from the HeLa cells, XJM5.1.1(+) and XEWS.2.3 cells, whereas no band was observed following amplification of the hamster B9 cells or the XJM5.1.1(−) cells. These results indicate that the cloned human genomic CII-3 sequences are present on chromosome 1, including on the portion of chromosome 1 comprising MAC8.2.3.

The complete human CII-3 gene readily can be determined by subcloning positive lambda genomic clones that have been isolated but not yet characterized. In addition, the complete human CII-3 gene can be obtained by rescreening the lambda DASH library to identify additional clones containing CII-3 sequences. In addition, a genomic library of XEWS.2.3 cells, including MAC-8.2.3, can be prepared and screened for genomic CII-3 sequences using the available cloned human genomic or cDNA CII-3 sequences (see Au et al., *Gene* 159:249–253 (1995), which is incorporated herein by reference). Portions of the human CII-3 gene sequences present in pJOS2 or the human CII-3 cDNA (SEQ ID NO: 1) can be used as probes to screen the library.

If the human CII-3 gene cannot be characterized completely from lambda genomic clones, a yeast artificial chromosome (YAC) or a "bacterial artificial chromosome" (BAC) containing the human CII-3 gene can be purchased from Genome Systems, Inc. (St. Louis, Miss.). Essentially, the manufacturer is provided with specific primers or a unique sequence to use as a probe. A sequence such as that shown as SEQ ID NO: 3, 5, 6 or 7 is ideal for this purpose, since these sequences do not encompass a coding region or regulatory region. The manufacturer then screens a BAC or YAC library, identifies a BAC or a YAC containing complementary sequences, and provides the YAC or BAC, the insert of which can be subcloned and characterized using routine methods.

Positive clones are selected and redundant clones are identified by restriction mapping. Unique clones are isolated, subclones are prepared, and the DNA sequences are determined. Overlapping sequences are identified and used to construct the entire human CII-3 gene sequence. This method allows the identification and isolation of isogenic sequences useful for targeted integration by homologous recombination (Tea Riele et al., *Proc. Natl. Acad. Sci., USA* 89:5128–5132 (1992), which is incorporated herein by reference).

To identify additional exons and, if present, introns, a combination of restriction mapping and partial sequencing is performed using the available cloned sequences as a probe. Putative unique sequences are examined by Southern blot analysis, comparing human genomic DNA and genomic DNA obtained from XEWS.2.3 cells. In particular, genomic sequences encoding the promoter and a portion of exon 1 will be identified.

EXAMPLE III

Modification of MAC-8.2.3

This example describes methods for introducing an exogenous nucleic acid sequence into MAC-8.2.3.

An exogenous nucleic acid sequence can be introduced into MAC-8.2.3 using homologous recombination (Ayares et al., supra, 1986; Capecchi, supra, 1989). Briefly, a construct is made containing the exogenous nucleic acid sequence of interest flanked on either side by nucleic acid sequences encoding the human CII-3 gene. In particular, unique sequences such as those identified, for example, as SEQ ID NOS: 3, 5, 6 and 7 are used such that the exogenous nucleic acid sequence is targeted to a specific locus (see FIG. 1A). Other unique sequences of the human CII-3 gene obtained as described in Example II also can be used to specifically target an exogenous nucleic acid sequence such as a selectable marker, a loxP site or a telomere sequence to the 5'-end or 3'-end of the human CII-3 gene, including at the site of human CII-3 promoter if it is desired that the exogenous nucleic acid sequence be expressed from the CII-3 gene promotor. Depending on the insertion site of the targeted sequence, CII-3 gene function can be disrupted, if desired.

An introduced exogenous nucleic acid sequence can be a loxP sequence or an FLP sequence. The introduction of such a site into MAC-8.2.3 and, in particular, into the CII-3 gene present in MAC-8.2.3 provides a means to readily introduce subsequent exogenous nucleic acid sequences into MAC-

8.2.3 in a site specific manner using the Cre recombinase from bacteriophage P1 or the FLP recombinase from *S. cerevisiae*, respectively. A loxP site, for example, is introduced into the CII-3 gene present on MAC-8.2.3. The loxP site is introduced by transfection of a targeting vector containing the neogene and promoter, flanked by two loxP sites, which, in turn, are flanked by human CII-3 gene sequences containing the CII-3 promoter on one side and CII-3 gene exon or intron sequences on the other side, which further is extended by the HSV-tk gene sequence (see FIG. 1A).

The gene encoding puromycin resistance also can be incorporated into the targeting vector, either in place of or in addition to another selectable marker such as neo (Skerjanc et al., *Mol. Cell. Biol.* 14:8451–8459 (1994); Vara et al., *Nucl. Acids Res.* 14:4617–4624 (1986), each of which is incorporated herein by reference). Use of the puromycin gene has advantages over the neogene because puromycin is significantly less expensive than G418. Also, the cDNA encoding the green fluorescent protein (GFP) can be used as a selectable marker (see Yeh et al., *Proc. Natl. Acad. Sci., USA* 92:7036–7040 (1995), which is incorporated herein by reference). GFP is particularly useful as a selectable marker because cells expressing GFP can be identified and, if desired, isolated using a fluorescence activated cell sorter (FACS).

Following transfection of the host cells with the targeting vector, cells are grown in medium containing G418 and gancyclovir. Cells that grow in this medium express the neogene, but do not express the HSV-tk gene and, therefore, are considered to have incorporated the targeting vector by homologous recombination into the human CII-3 gene (see FIG. 1B). Targeted integration is confirmed using PCR. This method produces MAC-(loxP-neo-loxP), which contains an active neogene, flanked by loxP sites, integrated downstream of the promoter of the CII-3 gene.

Mammalian cells containing MAC-(loxP-neo-loxP) are transfected with a vector expressing the Cre gene, wherein transient expression of the Cre gene results in precise and efficient excision of the neogene, leaving a single loxP site in the untranslated portion of exon I (see FIG. 1C) and producing MAC-(loxP). Similarly, the purified Cre protein can be introduced directly into MAC-1-containing cells using lipofection (Baubonis and Sauer, supra, 1993). Precise excision of the neogene is confirmed by PCR. MAC-(loxP) contains a single loxP site useful for targeting an exogenous nucleic acid sequence.

A promoterless neogene can be introduced into MAC-8.2.3 such that a transcript produced therefrom contains the upstream portion of exon 1 and the loxP sequence, which forms the 5' untranslated region of the neo transcript (see Jeannotte et al., *Mol. Cell. Biol.* 11:5578–5585 (1991); Charron et al., *Mol. Cell. Biol.* 10:1799–1804 (1990); Schwartzberg et al., *Proc. Natl. Acad, Sci., USA* 87:3210–3214 (1990), each of which is incorporated by reference). If desired, the translation start site of the CII-3 gene, which encodes the portion of the polypeptide that targets it to the mitochondria, can be deleted.

Cells carrying a single loxP site on the minichromosome are cotransfected with a circular targeting vector containing an exogenous nucleic acid sequence and a second loxP site (see FIG. 1D) and with an expression vector containing the Cre recombinase gene. Recombinase-mediated integration of the vector at the loxP site in the MAC inserts the exogenous nucleic acid sequence into the MAC (see FIG. 1E). The loxP sites are oriented, with respect to each other, so as to yield the desired orientation of the introduced sequence. The targeting vector can contain a promoterless cDNA sequence, which, following specific integration, is expressed from the CII-3 promoter. In addition, the targeting vector can contain a neo gene, with promoter, to allow selection. Alternatively, the neogene can be promoterless and can be separated from the exogenous nucleic acid sequence by a short sequence, including an internal ribosome entry site, IRES, (see FIG. 1D; Mountford and Smith, *Trends Genet.* 11:179–184 (1995), which is incorporated herein by reference). The CII-3 promoter directs expression of a dicistronic mRNA and neo resistance occurs only following specific integration.

A MAC containing a dominant selectable marker can be transferred into a variety of mammalian cells and cells containing the MAC can be identified. Selection for G418 resistance or puromycin resistance is powerful and allows the identification of cells containing the MAC even where there is a low efficiency of transfer. A MAC can be introduced into a mammalian recipient cell by somatic cell fusion with a host donor cell such as XEW8.2.3, which contains the MAC (Carine et al., supra, 1986).

Prior to fusion, the host cell containing the MAC can be irradiated at a dose that fragments the host cell chromosomes, such as the hamster chromosomes in XEW8.3.2, but spares the MAC, which is not hit due to its small size. The irradiated host cells then are fused to a mammalian cell line such as COS cells (monkey), 3T3 cells (mouse), or other cells including human cells or mouse embryonic stem (ES) cells and cells that grow under the appropriate selection conditions are obtained.

EXAMPLE IV

Method of Reducing the Size of a MAC

This example describes methods for producing a MAC or for reducing the size of a MAC such as MAC8.2.3.

A MAC can be produced by irradiation of normal chromosomes or minichromosomes at a dose that results in their fragmentation. Similarly, irradiation can be used to reduce the size of a MAC such as MAC8.2.3. For example, host cells containing MAC8.2.3 can be exposed to a dose that results in the MAC being hit one or a few times. Such a method was used to obtain MAC-8.2.3 from hybrid cell line XM12.2.3, which contains a larger MAC, and can be used, if desired, to select a MAC that is smaller than MAC-8.2.3. Alternatively, by randomly inserting a selectable marker in the genome of a cell, fragmenting the chromosomes, and fusing the cell with an intact cell, neomycin resistant hybrids can be obtained. By screening the resistant hybrids using the methods disclosed herein, a new MAC having a neogene inserted in a pericentric location can be obtained.

Fragmentation of chromosomes, minichromosomes or a MAC using telomerase associated truncation also can be used to produce a MAC or reduce the size of a MAC. For example, a telomere sequence consisting of repeated units of the sequence TTAGGG can be introduced into the region of the CII-3 gene present on MAC8.2.3 such that sequences distal to the telomere with respect to the centromere are lost from the MAC.

Site specific targeting of a telomere sequence is accomplished by homologous recombination using a targeting vector as described in Example III, except that the telomere sequence is substituted, for example, for the loxP site. Of course, if a loxP site first is placed into MAC8.2.3, the telomere sequence can be introduced into the site by incor-

porating the sequence into an appropriate vector (see FIG. 1E), introducing the vector into a host cell containing MAC8.2.3 and expressing the Cre recombinase in the cell (see Example III). The sequences in the targeting vector for directing an exogenous nucleic acid sequence into MAC8.2.3 by homologous recombination can be, for example, those disclosed herein as SEQ ID NOS: 3, 5, 6 or 7, since these sequences likely are unique sequences in the human genome and, therefore, in MAC8.2.3. The use of such unique sequences will preclude insertion of the targeting vector into the hamster chromosomes present in the host XEWS.2.3 cells.

EXAMPLE V

Production of Transgenic Mice

This example provides a method for producing transgenic mice by stably expressing a MAC containing a selectable marker in the mice.

Transgenic mice are created by introducing a MAC containing an exogenous nucleic acid sequence into embryonic stem (ES) cells, then microinjecting the ES cells into mouse embryos. Methods for culturing ES cells are well known in the art (see, for example, Kriegler, supra, 1990). Briefly, superovulation is induced by intraperitoneal injection of hormones using a 27G½ needle to deliver less than 200 μl hormone and the mice are mated. The pregnant females are anesthetized by inhalation with Metafane and sacrificed by cervical dislocation and fertilized embryos are removed from the oviduct. ES cells containing a MAC are selected and microinjected into the embryos. Alternatively, a MAC is microinjected into an ovum, which is fertilized. Pseudopregnant females are anesthetized by inhalation with Metafane and the embryos or fertilized ova are implanted into the oviduct. Offspring, which are weaned at three or more weeks of age, are anesthetized by inhalation with Metafane, one half inch of tail is removed using a sterile blade and a blood sample is obtained. DNA is isolated from the blood sample and screened by Southern blot analysis to identify animals containing the exogenous nucleic acid.

Although the invention has been described with reference to the examples provided above, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 16

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1315 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: cDNA

(i x) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 27..536

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
ACTTCCGTTC CAGACCGGAA CCCAAG ATG GCT GCG CTG TTG CTG AGA CAC GTT      53
                             Met Ala Ala Leu Leu Leu Arg His Val
                              1               5

GGT CGT CAT TGC CTC CGA GCC CAC TTT AGC CCT CAG CTC TGT ATC AGA     101
Gly Arg His Cys Leu Arg Ala His Phe Ser Pro Gln Leu Cys Ile Arg
 10                  15                  20                  25

AAT GCT GTT CCT TTG GGA ACC ACG GCC AAA GAA GAG ATG GAG CGG TTC     149
Asn Ala Val Pro Leu Gly Thr Thr Ala Lys Glu Glu Met Glu Arg Phe
                 30                  35                  40

TGG AAT AAG AAT ATA GGT TCA AAC CGT CCT CTG TCT CCC CAC ATT ACT     197
Trp Asn Lys Asn Ile Gly Ser Asn Arg Pro Leu Ser Pro His Ile Thr
             45                  50                  55

ATC TAC AGT TGG TCT CTT CCC ATG GCG ATG TCC ATC TGC CAC CGT GGC     245
Ile Tyr Ser Trp Ser Leu Pro Met Ala Met Ser Ile Cys His Arg Gly
         60                  65                  70

ACT GGT ATT GCT TTG AGT GCA GGG GTC TCT CTT TTT GGC ATG TCG GCC     293
Thr Gly Ile Ala Leu Ser Ala Gly Val Ser Leu Phe Gly Met Ser Ala
     75                  80                  85

CTG TTA CTC CCT GGG AAC TTT GAG TCT TAT TTG GAA CTT GTG AAG TCC     341
Leu Leu Leu Pro Gly Asn Phe Glu Ser Tyr Leu Glu Leu Val Lys Ser
```

-continued

```
90                  95                 100                 105

CTG TGT CTG GGG CCA GCA CTG ATC CAC ACA GCT AAG TTT GCA CTT GTC      389
Leu Cys Leu Gly Pro Ala Leu Ile His Thr Ala Lys Phe Ala Leu Val
            110                 115                 120

TTC CCT CTC ATG TAT CAT ACC TGG AAT GGG ATC CGA CAC TTG ATG TGG      437
Phe Pro Leu Met Tyr His Thr Trp Asn Gly Ile Arg His Leu Met Trp
        125                 130                 135

GAC CTA GGA AAA GGC CTG AAG ATT CCC CAG CTA TAC CAG TCT GGA GTG      485
Asp Leu Gly Lys Gly Leu Lys Ile Pro Gln Leu Tyr Gln Ser Gly Val
    140                 145                 150

GTT GTC CTG GTT CTT ACT GTG TTG TCC TCT ATG GGG CTG GCA GCC ATG      533
Val Val Leu Val Leu Thr Val Leu Ser Ser Met Gly Leu Ala Ala Met
155                 160                 165

TGAAGAAAGG AGGCTCCCAG CATCATCTTC CTACACATTA TTACATTCAC CCATCTTTCT    593

GTTTGTCATT CTTATCTCCA GCCTGGGAAA AGTTCTCCTT ATTTGTTTAG ATCCTTTTGT    653

ATTTTCAGAT CTCCTTGGAG CAGTAGAGTA CCTGGTAGAC CATAATAGTG GAAAAGGGTC    713

TAGTTTTCCC CTTGTTTCTA AAGATGAGGT GGCTGCAAAA ACTCCCCTTT TTTGCCCACA    773

GCTTGCCTAC TCTCGGCCTA GAAGCAGTTA TTCTCTCTCC ATATTGGGCT TTGATTTGTG    833

CTGAGGGTCA GCTTTTGGCT CCTTCTTCCT GAGACAGTGG AAACAATGCC AGCTCTGTGG    893

CTTCTGCCCT GGGGATGGGC CGGGTTGGGG GGTGGGTTGG GTGAAGCTTT GGGTTGCCAC    953

TGCCTGTGGG TTTGCTGGCT TAAAGGACAA TTCTCTTTCA TTGGTGAGAG CCCAGGCCAT    1013

TAACAACTAA CACAGTGTTA TTGAAAGAAG AGAGGTGGGG GTGGAGGGGA ATTAGTCTGT    1073

CCCAGCTAGA GGGAGATAAA GAGGGCTAGT TAGTTCTTGG AGCAGCTGCT TTTGAGGAGA    1133

AAATATATAG CTTTGGACAC GAGGAAGATC TAGAAAATTA TCATTGAACA TATTAATGGT    1193

TATTTCTTTT TCTTGGATTT CCAGAAAAGC CTCTTAATTT TATGCTTTCT CATCGAAGTA    1253

ATGTACCCTT TTTTCTGAA ACTGAATTAA ATACTCATTT TAAAAAAAAA AAAAAAAAAA    1313

AA                                                                  1315
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 169 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Ala Ala Leu Leu Leu Arg His Val Gly Arg His Cys Leu Arg Ala
 1               5                  10                  15

His Phe Ser Pro Gln Leu Cys Ile Arg Asn Ala Val Pro Leu Gly Thr
            20                  25                  30

Thr Ala Lys Glu Glu Met Glu Arg Phe Trp Asn Lys Asn Ile Gly Ser
        35                  40                  45

Asn Arg Pro Leu Ser Pro His Ile Thr Ile Tyr Ser Trp Ser Leu Pro
    50                  55                  60

Met Ala Met Ser Ile Cys His Arg Gly Thr Gly Ile Ala Leu Ser Ala
65                  70                  75                  80

Gly Val Ser Leu Phe Gly Met Ser Ala Leu Leu Leu Pro Gly Asn Phe
                85                  90                  95

Glu Ser Tyr Leu Glu Leu Val Lys Ser Leu Cys Leu Gly Pro Ala Leu
            100                 105                 110

Ile His Thr Ala Lys Phe Ala Leu Val Phe Pro Leu Met Tyr His Thr
```

-continued

```
                115                 120                 125
Trp Asn Gly Ile Arg His Leu Met Trp Asp Leu Gly Lys Gly Leu Lys
    130                 135                 140
Ile Pro Gln Leu Tyr Gln Ser Gly Val Val Val Leu Val Leu Thr Val
145                 150                 155                 160
Leu Ser Ser Met Gly Leu Ala Ala Met
                165
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 257 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
TTCCCCATAC TCAGGAGGCT GAAGCAGGAG ACTCGCTTGA ACTTGGGAGG TGGAGGTTGC   60
AGTGAGCCAA GATTGCACCA ATATACTCCA GCCTGGGTGA CAGAATGAGA CTCTGTCTCA  120
AGAAAAAAAG AAAACAAAAA TCTTCTCCAT TTCAAAATGG TTTAGAATTG TATGAGGTGC  180
CAGGGGTCCC AGTTTTATGT ATCATATTAG TTGTAACTTA TGAGCAGCTG TGACAAGCTA  240
CTTGGTTTTC TCCTCAG                                                 257
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 164 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
GGGTCTCTCT TTTTGGCATG TCGGCCCTGT TACTCCCTGG GAACTTTGAG TCTTATTTGG   60
AACTTGTGAA GTCCCTGTGT CTGGGGCCAG CACTGATCCA CACAGCTAAG TTTGCACTTG  120
TCTTCCCTCT CATGTATCAT ACCTGGAATG GGATCCGACA CTTG                    164
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 173 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
GTAAGTTAAT TCGGGATTTG CACATTTTCT CTGTGAAGGG AGTGGGGAGA CTGGGAGGAT   60
TCTTTCCTTC ATTACTGGGT TTAGTGCTGT TCTTTTTTTT TTTTCCCAAG AGTGGAGTGT  120
CTCGCTCTAT TGCCCAGGCT GGAGTGCAGT GGTGCGATCT CAGCTCACTG CAA         173
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 327 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
AATTAACCCT CACTAAAGGG AGTCGACTCG ATCCCAAGTA GTCTGTCTCC CATCATAAAC   60
TTGAACATGA GTTTAAATCT TCTCCTTTTC AAGGCCGGGT GCAGTGGCTC ACACCTGTAA  120
```

-continued

```
TCTCAGCACT TTGGGAGGCA AAGGTGGGCA GATCACTTGA GGTCAGGAGT TCGAGACCAG    180
CCTGGCCAAC TTGGTGAAAC CCTGTCTCTA CTAAAAATAC AAAAATTAGC TGGGCGTTGT    240
GGTGGGCACC TGTCATCCCC ACTACTCAGG AGGCTGAAGC AGGAGACTCG CTTGAAACTT    300
GGGAAGTGGA AGTTGCAGTG AACCCAA                                        327
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 383 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
TTGGCACCCA GTTTCTATTA AAGTTGGCCC AATTCTGTCC AACATCTCAA AACAGAAATG     60
CAATATAATG TGTAGATAAG AAAAGGTAAT CTATTTGAGT CCTGTCAGAA GCACTACTCT    120
GGGTCAACAG GAACGGAAGA ATGAAAGCAG CAACAATGGT TATCTAGCTC ATAACTGAAT    180
CCCCAGTGTC TACAACAGTA CCTGACACAT AAATAGGTAC CAATTAATAT TTATGTCATA    240
AACATGCATT CTATGCCTTC AGGGATCTCT TTTAAATATC CCTCTTAAAA ATGAAGAGTT    300
CAGCAGGGCA CAGTGGCTCA CGTCTGTAAT CCTAGCACTT TGGGAAGCTG AAAAGGGTGG    360
ATCACAAGGT CAGATTTGAA AAA                                            383
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
TGGTGAAACC CTGTCTCTAC                                                20
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
TCTATGCCTT CAGGGATCTC                                                20
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
ACTTGTGAAG TCCCTGTGTC                                                20
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

-continued

(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AAOTGTCGGA TCCCATTCCA 20

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GACTCGAGTC GACATCGATT TTTTTTTTT TTTTT 35

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GCCAGCCCCA TAGAGGACAA CAC 23

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GACTAGTCGA CTGCAGGGGG GGGGGGGGGG 30

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TGCCAGCCCT ACAGAGGACA ACAC 24

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CTGGAGTAAG AACACTACTT TAAACCGTCC 30

60

I claim:

1. A mammalian artificial chromosome (MAC) consisting of MAC-8.2.3 contained in host cell line XEW8.2.3, wherein said cell line has been deposited as Accession No. ATCC CRL 11992.

2. A MAC comprising MAC-8.2.3 and a unique cloning site, wherein the MAC is less than about 0.1% of the size of the normal haploid genome of the mammalian cell from which the centromere was obtained.

3. The MAC of claim 2, wherein said unique cloning site comprises a nucleic acid sequence encoding a selectable marker.

4. The MAC of claim 2, wherein said unique cloning site comprises a portion of a nucleic acid encoding a CII-3 gene.

5. The MAC of claim 2, wherein said unique cloning site comprises a portion of a nucleic acid sequence encoding a human CII-3 gene selected from the group consisting of Seq ID No. 3, Seq. ID No. 4., Seq. ID No. 5, Seq. ID No. 6, Seq. ID No. 7, and a portion of Seq ID No. 1.

6. The MAC of claim 3, wherein said selectable marker is an exogenous nucleic acid sequence.

7. An isolated mammalian cell containing the MAC of claim 2.

8. The mammalian cell of claim 7, wherein said cell is a human cell.

9. An isolated mammalian cell containing the MAC of claim 6, wherein said cell stably expresses said exogenous nucleic acid sequence.

10. The mammalian cell of claim 31, wherein said cell is a human cell.

11. A method of preparing a MAC from MAC 8.2.3, comprising the steps of:

(a) fragmenting MAC 8.2.3, and

(b) selecting a centromeric fragment of said MAC 8.2.3, wherein said centromeric fragment contains less than about 0.1% of the DNA present in a normal haploid cell from which said MAC 8.2.3 was obtained.

12. The method of claim 11, further comprising the step of first inserting an exogenous nucleic acid sequence encoding a selectable marker into said MAC 8.2.3.

13. The method of stably expressing a selectable marker in a mammalian cell, comprising introducing MAC 8.2.3 containing said selectable marker into said cell and stably expressing said selectable marker in said cell.

14. The method of claim 13, wherein said selectable marker is an exogenous nucleic acid sequence.

15. The method of claim 13, wherein said mammalian cell contains a mutation and said selectable marker complements said mutation.

16. The method of claim 13, wherein said introducing comprises fusing a host donor cell containing said MAC 8.2.3 with a recipient cell, thereby producing a hybrid cell containing said MAC 8.2.3, wherein said selectable marker is stably expressed in said hybrid cell.

17. The method of producing an exogenous mammalian gene product in a mammalian cell, comprising the steps of:

(a) introducing MAC 8.2.3 containing an exogenous nucleic acid sequence encoding the mammalian gene product into said mammalian cell, and

(b) expressing said mammalian gene product in said mammalian cell.

* * * * *

EXHIBIT 5

Am. J. Hum. Genet. 52:598–608, 1993

Molecular Basis for the Polymorphic Forms of Human Serum Paraoxonase/Arylesterase: Glutamine or Arginine at Position 191, for the Respective A or B Allozymes

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Summary

The paraoxonase/arylesterase gene is located close to the cystic fibrosis gene on chromosome 7. Human serum contains two paraoxonase/arylesterase allozymes, A and B, which differ in their substrate specificities and kinetic properties. Purified A, AB, and B esterases were digested with trypsin, and the resultant peptides were compared by high-performance liquid chromatography. The elution profiles were very similar for all three samples, except for (1) one peptide (i.e., peptide A) seen only in the A and AB profiles and (2) another peptide (i.e., peptide B) seen only in the B and AB profiles. Sequencing revealed that peptide A had glutamine at amino acid position 191, whereas peptide B was generated by cleavage on the carboxy side of position 191, presumably because there was a basic (trypsin-specific) amino acid at that position. Working independently, our laboratory and one other laboratory have sequenced the coding region for paraoxonase from human liver cDNA libraries and have identified two polymorphic sites: Arg/Gln at position 191 and Leu/Met at position 54. Using PCR amplification and direct sequencing of nucleotides in both polymorphic regions with genomic DNA, we have estimated the allelic frequencies and have determined their concordance with the serum paraoxonase allozyme phenotypes in 27 unrelated adults and in 16 members of a three-generation pedigree. Among unrelated individuals, the Met/Leu polymorphism at position 54 did not correlate with the serum esterase phenotype. In contrast, the particular amino acid at position 191 correlated perfectly with serum phenotypes: A-type individuals had Gln at position 191, and B-type individuals had Arg at position 191; AB-type serum was found only with the heterozygous (Arg/Gln) combination. Pedigree analysis showed both polymorphisms to be inherited in the expected Mendelian manner and confirmed that only the 191 polymorphism showed concordance with the serum paraoxonase/arylesterase phenotypes.

Introduction

Human serum paraoxonase/arylesterase catalyzes the hydrolysis of organophosphates, aromatic carboxylic acid esters, and carbamates, but its physiological function is still unknown (see the recent general review by La Du [1992]). The enzyme has been highly purified from human serum, in this laboratory (Gan et al. 1991), and the characteristics of the two allozymic forms have been described (Smolen et al. 1991).

The genetic basis of the paraoxonase polymorphism was first carefully investigated by Playfer et al. (1976). They concluded that high and low serum paraoxonase activities were controlled by two alleles at a single autosomal locus. The two allozymes are presumed to be products of a gene (i.e., PON) located close to the cystic fibrosis gene on the long arm of chromosome 7 (Tsui et al. 1985). Several years ago, our laboratory developed a method for identifying A, AB, and B serum paraoxonase/arylesterase phenotypes (Eckerson et al. 1983*a*, 1983*b*). Advantage was taken of the observations that

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the two allozymes differ in qualitative properties: the B allozyme has a relatively higher paraoxon-hydrolyzing activity and shows a greater degree of stimulation of its paraoxon-hydrolyzing activity by 1 M NaCl than does the A allozyme (Eckerson et al. 1983*b*). The quotient obtained by dividing paraoxonase activity in the presence of 1 M NaCl by the arylesterase activity (measured with phenylacetate), called the "ratio" of these activities, has been used to diagnose the phenotype of serum samples (Eckerson et al. 1983*b*). Chlorpyrifos oxon has also been suggested as a substitute for phenylacetate for serum phenotyping (Furlong et al. 1988; Hassett et al. 1991). The designated A, AB, and B esterase phenotypes are based on the ratios with the two substrates and follow the expected autosomal inheritance patterns; no exceptions have ever been noted. The above genetic evidence strongly supports the view that either the two activities are properties of the same enzyme or the genes for the two enzymes must be very closely linked. Basic questions about (1) how the structural differences in the A and B allozymes account for their distinctive catalytic properties and (2) whether the allozymes both have paraoxonase and arylesterase activities have been under study for several years in our laboratory.

This report describes experiments on purified A and B paraoxonase allozymes to identify the structural differences in the allozymic proteins, as well as molecular studies on human paraoxonase genes to determine the DNA basis for the paraoxonase polymorphism. Protein sequencing has revealed the possible occurrence of an intramolecular disulfide bond, the location of one asparagine-linked carbohydrate, and the first amino acids in the mature enzyme protein. We independently cloned the paraoxonase cDNA coding for amino acid methionine 11, to the last amino acid in the protein, and confirmed the full-length cDNA sequence recently reported by Hassett et al. (1991). Amino acids methionine 1 to glycine 10 were recloned from the same cDNA library used by Hassett et al. (1991). The location and some sequence data about two introns adjacent to the two polymorphic sites are described in the present paper, since both were used in our laboratory for PCR amplification and direct sequencing of the polymorphic regions. Two common polymorphic sites at amino acids 54 and 191 (these same positions are designated "55" and "192," respectively, by Hassett et al. 1991) have been identified, so it has been necessary to compare each person's serum paraoxonase phenotype with their DNA-structural polymorphisms in 27 unrelated individuals and in members of a three-generation pedigree, to determine whether one or both of the structural polymorphisms cosegregate with the serum paraoxonase phenotypic characteristics. We found that only the polymorphism at amino acid position 191 showed the expected correlation between paraoxonase genotypes and phenotypes.

Material and Methods

Paraoxonase Purification and Amino Acid Sequencing

Paraoxonase/arylesterase was purified from frozen human serum that had been previously phenotyped according to a method described elsewhere (Eckerson et al. 1983*b*) and is summarized in the Phenotyping subsection below. Several units of serum, usually four, from different individuals of the same A, AB, or B phenotype were pooled and purified by the method developed in our laboratory (Gan et al. 1991); serum was treated with a blue agarose column, washed with salt buffer, and eluted with detergent buffer. Active fractions were fractionated on a DEAE biogel column, washed with detergent buffer, and finally eluted with a salt gradient. Most preparations were carried through a second DEAE-agarose-column purification step and were estimated to be over 50% pure enzyme, on the basis of the specific activities obtained, and to be over 90% enzyme protein, on the basis of the PAGE gel patterns of the stained protein bands (Gan et al. 1991). The purified enzyme has a molecular weight of about 43.0 kDa. A purification scheme for rabbit and human serum paraoxonases has been described by Furlong et al. (1991). Amino acid sequencing of peptides obtained by proteolytic digestion of purified enzyme revealed that human apolipoprotein was a minor contaminant in our preparations, but no other contaminants were identified. Non-apolipoprotein A-I sequences were assumed to represent paraoxonase protein, and these were later identified in the protein sequence translated from the cDNA nucleotide sequence.

The purified paraoxonase preparations were digested with trypsin or pepsin, and the resultant peptides were characterized and separated by high-performance liquid chromatography (HPLC) as described by Lockridge et al. (1987). Some of these peptides were sequenced manually by the Edman degradation method (Tarr 1982), followed by quantitation of the derivatized amino acids by HPLC (Black and Coon 1982). Sequence searches and comparisons with known protein and DNA sequences were carried out by using the facilities of Protein Identification Resource (Georgetown University, Washington, DC), Intelligenetics (Mountain View, CA), and Genetics Computer Group (Madison).

Genbank (Cambridge, MA) was used for homology searches.

A sample of undigested purified paraoxonase/arylesterase was submitted, for automated amino acid sequencing, to the University of Michigan Protein and Carbohydrate Structure Facility, to verify the amino acid sequence at the N-terminus of the mature protein. Since this nonapolipoprotein sequence showed that the first amino acid was alanine, the latter was designated as "residue number 1" of the paraoxonase amino acid sequence. The first base of the codon for that initial alanine was identified as "nucleotide number 1" in our numbering system for the cDNA and genomic DNA sequences (fig. 1). Hassett et al. (1991) concluded that the amino acid (i.e., methionine) preceding what we have named "ala 1" was the first amino acid in the nascent paraoxonase protein, and so Hassett et al.'s numbering begins by designating that initiator methionine as "amino acid 1." The University of Michigan Protein and Carbohydrate Structure Facility also analyzed a number of the isolated peptides for both the amino acid sequences and carbohydrate components.

Cloning and Sequencing of the Paraoxonase cDNA Clones

A human liver cDNA library was provided by Dr. S. Woo (Kwok et al. 1985). This library was screened by standard in situ hybridization techniques (Maniatis et al. 1982). The oligonucleotide probes employed were either mixtures of oligonucleotides to include all the possible codon compositions or unique-sequence oligonucleotide probes constructed on the basis of tryptic digest peptide sequences obtained from purified paraoxonase. The codons selected for the unique-sequence probes were those most frequently used for those amino acids in human proteins (Lathe 1985). Lambda gt11 clones were subcloned into M13 sequencing vectors, and these were sequenced with universal and specific primers (Sanger et al. 1980).

By use of random-labeled probes (Feinberg and Vogelstein 1983) generated from our first clones, the Woo library was rescreened by in situ hybridization techniques. Hybridizing plaques were characterized by PCR amplification without plaque purification or DNA isolation (Hamilton et al. 1991). Lambda gt11 primers were purchased from New England Biolabs.

To examine the Woo library specifically for clones extending as far as possible in the 5′ direction, an aliquot of the library was amplified by the PCR technique (Hamilton et al. 1991) by pairing vector primers and paraoxonase primers located near the N-terminal sequence of paraoxonase. The products were directly sequenced with nested paraoxonase primers extending toward the N-terminal end of the protein. Another human liver cDNA library, HL1001b, purchased from Clontech, was analyzed in a similar manner.

Several introns were located by PCR amplification of genomic DNA with cDNA primers. Genomic PCR products were examined to see whether any were appreciably larger than expected; these would probably contain intron sequences. A number of these were sequenced to determine both whether the sequences were indeed from the paraoxonase gene and exactly where introns were located. Both a 1.0-kb intron in the region coding for amino acid 66 and a 1.6-kb intron in the region coding for amino acid 234 were found (fig. 1).

Blood and DNA Samples

Blood samples (10–20 mL) were collected from 24 unrelated volunteers and were also recollected from 16 members of a three-generation pedigree that we had previously analyzed, in 1988, for the atypical (dibucaine-resistant) variant of butyrylcholinesterase (McGuire et al. 1989). Three unrelated individuals from this pedigree (II-6, I-1, and I-2, who had genotypes AA, AB, and BB, respectively) have also been included, to make the total of 27 unrelated individuals. DNA was isolated from the buffy coat of the blood samples by the salt-chloroform extraction method of Mullenbach et al. (1989).

PCR Amplification of the Polymorphic Sites

Genomic DNA sequences in the regions coding for the two polymorphic sites both occur close to introns, so one of each pair of the PCR amplification primers was selected to be an intron-region primer. Thus, pairs of one intron primer and one exon primer were used for the regions corresponding to amino acids 54 and 191. PCR amplification products, approximately 180 and 230 bp, respectively, were obtained individually or in the same amplification mixture, for direct sequencing with suitable intronic primers (fig. 1).

Phenotyping

The quotient of paraoxonase activity measured at pH 10.5 with 1 M NaCl in the reaction mixture, divided by the arylesterase activity with phenylacetate, was calculated as described elsewhere (Eckerson et al. 1983*b*; Gan et al. 1991). The ratio of these activities was used to determine individual phenotypes, as follows: type A, ratio 1.21 ± 0.19; type AB, ratio 4.68 ± 0.85;

and type B, ratio 8.36 ± 0.70 (data are mean ± SD). Phenotype ranges are 0.9–2.5, 2.6–7.5, and 7.6–12.0, respectively, for the A-, AB-, and B-type allozymes.

Results

Enzyme Purification and Peptide Analyses

Purified paraoxonase/arylesterase preparations were used to determine the N-terminal amino acid residues, to gain information about the secondary structure of the enzyme, and to obtain sequence information for later use to clone the paraoxonase gene. Our early cDNA cloning revealed that about half of the amino acid residues of the mature protein were included in the sequencing of the 13 tryptic and peptic digest peptides obtained from the purified enzyme. Nonparaoxonase sequences from our protein preparations were all from apolipoprotein A-I.

Samples of purified paraoxonase were subjected to nonreducing trypsin or pepsin digestion, followed by isolation and purification of the resulting peptides by HPLC. A few of the fractions sequenced in the earlier experiments were obviously contaminants, derived from human apolipoprotein A-I. However, most peptides sequenced had no matches in the available computerized sequence banks and were used later in designing oligonucleotide probes to clone the paraoxonase gene. One tryptic peptide gave two simultaneous sequences, and it was resolved by reduction into two constituent peptides that were then separated and individually sequenced. Each contained a cysteine residue (residues 32–45 and residues 349–354) (fig. 1). The shorter peptide (residues 349–354) of the two was believed to be the carboxyl terminus of the protein, because of the complete absence of additional amino acids after Leu 354 in subsequent sequencing cycles; the other peptide in the disulfide linkage (cysteines 41 and 352) was later located near the amino terminus of the protein. The carboxy termini and amino termini of native paraoxonase may be in disulfide linkage through cysteines 41 and 352. One peptide (residues 250–259) contained a carbohydrate moeity, later shown to be linked to asparagine 252 (fig. 1). Subsequently cDNA sequencing revealed several additional possible sites for N-glycosylation. The carbohydrate content of the purified enzyme was found to be 15.8% (Gan et al. 1991).

Sequence analysis of undigested enzyme showed the first four residues of the mature serum paraoxonase to be alanine, lysine, leucine, and isoleucine, and this sequence was subsequently confirmed by the DNA nucleotide sequences. The ease of sequencing the undigested enzyme isolated by the DEAE agarose II step indicated that the apparent blockage of the N-terminal amino acid that we noted earlier (Gan et al. 1991) probably resulted from the extra steps used in the isolation procedure at that time, such as extraction of the purified protein from acrylamide gel.

Allozymic Differences in Peptides

Tryptic digest peptide profiles after HPLC analysis from purified preparations of A, AB, and B phenotype samples showed distinctive patterns (fig. 2). Although the three profiles were very similar, one unique peak (peptide A) was present in the preparations of A and AB samples, and another unique peak (peptide B) was seen only in the profile of B and AB samples. Peptides A and B had retention times of 40 and 44 min, respectively. Peptides A and B were collected in two subsequent HPLC runs, and the amino acid sequences were determined (fig. 3). These obviously were not alternative sequences at the same location, and their relationship did not become clear until some months later, after we had cloned the paraoxonase/arylesterase cDNA and had translated the nucleotide sequence to amino acids. It was then obvious that the two peptides were adjacent and differed in amino acid residue number 191 (fig. 3). Peptide B represented roughly the second half of peptide A. Since peptide A had Gln at position 191, no tryptic cleavage would occur between residues 191 and 192 in this long peptide. In peptide B, however, the first residue was number 192, and residue 191 should be either arginine or lysine, to create a tryptic cleavage site. Amino acid 191 was found to be Arg, in our cDNA library sample. It seemed possible that Gln/Arg at position 191 might be the critical feature determining the A/AB/B phenotypic polymorphism.

Cloning of Human Paraoxonase/Arylesterase

The entire paraoxonase coding region was sequenced by using a combination of different methods. The Woo human liver cDNA library did not extend in the 5′ direction any farther than the codon for residue 11 (methionine); however, it was very useful for sequencing all of the remaining coding sequence in the 3′ direction—i.e., through residue 354, the last amino acid of the mature protein, and to the polyadenylation sequences. The Woo cDNA library in lambda gt11 was initially screened with oligonucleotide probes PX2 and PX5 (fig. 1), as described above under Material and Methods. Two overlapping clones were isolated and analyzed; clone W-II-14 extended from nucleotide 150 to nucleotide 1646; it represented amino acids 50–354

Figure 1 Human serum paraoxonase/arylesterase cDNA showing sequenced peptides and the oligonucleotides used to characterize the gene and polymorphic sites. The location of two introns and the DNA sequence of the introns adjacent to the intron-exon junctions are

and extended 584 nucleotides beyond the coding region in the 3′ direction. Clone W-II-12 contained nucleotides 300–1308, representing amino acid residues 100–354, and it extended 246 nucleotide bases 3′ beyond the last amino acid. No differences were found in the nucleotide sequences in the overlapping segment of these clones.

The Woo library was also screened with a random-labeled cDNA probe derived from clone W-II-14, which represented the 5′ portion of the cloned cDNA. Eighteen hybridizing plaques were found. These non-plaque-purified clones were analyzed by direct PCR of heat-denatured viral particles, to find those that might contain additional coding sequence toward the 5′ end. First, clones were amplified with human paraoxonase primers encompassing amino acids 50–188 (bases 150–564), with primers PX7 and PX6. Clones yielding PCR products of approximately 400 bases were further analyzed. These were amplified with lambda gt11 primers (forward or reverse primers) in combination with primer PX6. Clones having additional 5′ coding sequence would produce fragments larger than the PX6/PX7 product. Clone W-IV-10 gave the longest PCR product, and it was directly sequenced by using paraoxonase primer PX8. Clone W-IV-10 coded for 21 additional amino acids and allowed the DNA sequence corresponding to amino acids 29–50 to be identified.

Direct amplification of an aliquot of the Woo library as described under Material and Methods gave the DNA sequence for the region coding for amino acids 10–29, but there appeared to be no clones extending in the 5′ direction any farther than the codon for amino acid 10. Therefore, another human liver cDNA library, HL1001b, was used to obtain the nucleotide sequence extending in the 5′ direction to the −4 codon, by direct PCR amplification and sequencing. No clones extending in the 5′ direction past the −4 codon were found in this library. The HL1001b library was used by Hassett et al. (1991) for their sequencing of paraoxonase cDNA clones, and they also found no clones extending 5′ beyond the −4 codon when they used in situ hybridization techniques with this cDNA library.

Polyadenylation sites were found at two locations: 246 and 584 nucleotide bases downstream from the last amino acid codon in the Woo cDNA library. Hassett et al. (1991) found three polyadenylation sites to be present in clones from the HL1001b cDNA library.

Polymorphic Sites

We examined both polymorphic sites in a population sample by direct sequencing after PCR amplification of those regions, to verify the structural mutations of primary interest, and we compared the neighboring nucleotides in these individuals. Genomic DNA samples from 27 unrelated people and from family members of a three-generation pedigree were sequenced to determine the allelic frequencies and inheritance patterns of both polymorphisms, as well as their concordance with the individual serum paraoxonase/arylesterase phenotypes.

Results from the tests in 27 unrelated individuals are shown in table 1. It is clear that the polymorphism at position 54 does not correlate at all with the paraoxonase phenotype, but the polymorphism at position 191 does. All the A-phenotype individuals were homozygous for glutamine at this position. The other phenotypes were equally in agreement with corresponding genotypes; those homozygous for arginine had B-phenotype esterase, and all those heterozygous (AB phenotype) had glutamine/arginine at the polymorphic site. The amino acid at position 54 in individuals homozygous for glutamine at amino acid 191 did not correlate with serum arylesterase activity, serum paraoxonase activity (with or without 1 M NaCl), or quotients calculated from these three activities. Further studies will be needed to find out how the allozymes can be influenced by the particular amino acids at position 54.

The results from the pedigree analysis (fig. 4) were equally unequivocal about the correlation of the paraoxonase/arylesterase phenotypes with the polymorphism at position 191. In contrast, the amino acids at position 54 did not correlate with the serum esterase polymorphism. The same associations were seen in relationships between the amino acids at position 191 and the serum enzyme phenotypes in the family pedigree as were seen in the collection of unrelated individ-

indicated. Bars above the amino acid sequences are those identified as tryptic or peptic digest peptides that were sequenced directly. An asterisk above an amino acid (see Arg17, His19, and Ala216) means that it did not agree with the cDNA. Codons for these sequenced amino acids disagree, by one nucleotide, with the cDNA (shown) and could be additional polymorphic sites. Asn269, though a theoretical site of N-glycosylation (Bause 1983), was not found to be glycosylated when the peptide encompassing amino acids 267–281 was sequenced. Asn226 and Asn323 are also possible sites of N-glycosylation. PX7 with PX12 and PX9 with PX16 revealed the presence of 1.0- and 1.6-kb introns, respectively, when used to amplify genomic DNA. PX21, PX20, PX22, PX15, PX28, and PX26 were used to amplify and sequence the two polymorphic sites. PX12, PX8, PX4, or PX18 was paired with lambda gt11 primers to amplify aliquots of cDNA libraries directly.

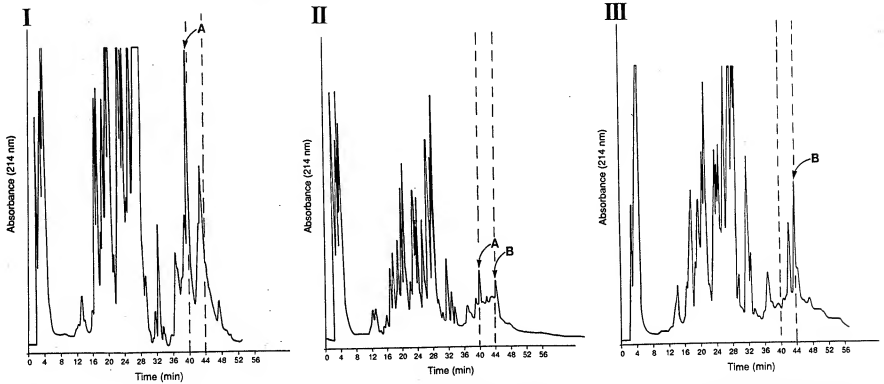

Figure 2 HPLC profiles of tryptic digests of purified serum paraoxonase from pooled serum of A-type individuals (I), pooled serum of AB-type individuals (II), and pooled serum of B-type individuals (III). Peptides A and B are shown. Retention times are indicated by dashed lines, for clarity (method of Mercer et al. 1986). Note that panels I–III each represent different amounts of total protein and therefore cannot be compared directly. For instance, panel I represents a relatively large amount of protein, and the shoulder at 44 min appears significant. However, consider that most peaks are off the scale and that the shoulder at 44 min is less than one-third as tall as the distinct A peak in the same panel. Also, notice that a peak at a retention time of 43 min occurs in all three profiles. This 43-min peak is significantly shorter than either the A peak or the B peak, in all three profiles. In profile I this 43-min peak is prominent and half the height of the profile I A peak; however, there is clearly no prominent B peak at the retention time of 44 min.

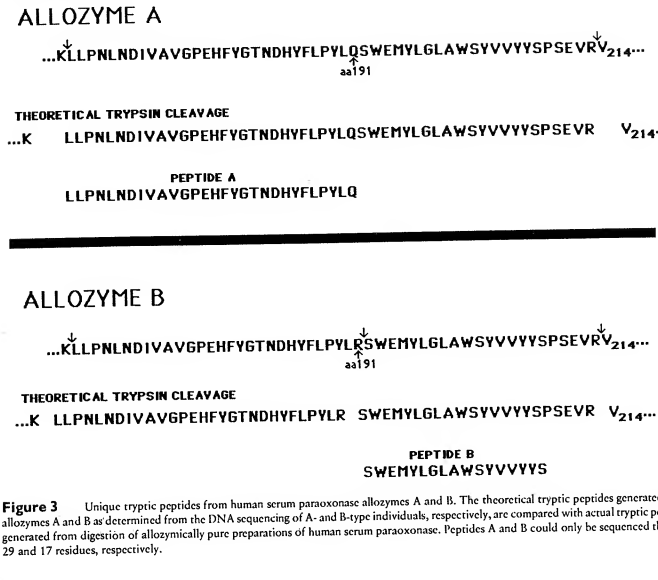

Figure 3 Unique tryptic peptides from human serum paraoxonase allozymes A and B. The theoretical tryptic peptides generated from allozymes A and B as determined from the DNA sequencing of A- and B-type individuals, respectively, are compared with actual tryptic peptides generated from digestion of allozymically pure preparations of human serum paraoxonase. Peptides A and B could only be sequenced through 29 and 17 residues, respectively.

uals. It appears likely that the polymorphism at position 191 affects in some way the structural properties of the enzyme, which confer the distinctive catalytic properties to the A, AB, and B types of paraoxonase/arylesterase.

Discussion

The structural basis for each of the two polymorphic sites is shown in figure 1. The Met/Leu polymorphism at position 54 depends on ATG/TTG, and the Arg/Gln polymorphism at position 191 depends on CGA/CAA. Both polymorphic sites involve base changes that theoretically could be detected as RFLPs. If *Nla*III were used, the Met sequence CATG would be cut, but the CTTG sequence for Leu would not be cut; and *Alw*I would cut the sequence (C)GATCC of Arg but would not cut the corresponding (C)AATCC sequence of Gln. However, we elected to use direct sequencing of PCR products. The data presented here offer evidence that the polymorphic human serum paraoxonase/arylesterase phenotype is associated with the Arg/Gln structural polymorphism at amino acid position 191. We conclude that the A allozyme has glutamine at this position and that the B allozyme has arginine at this position. This difference in structure of the allozymes also explains why the B form has an additional trypsin cleavage site, and this would account for the unique A and B peptide peaks observed after tryptic digestion of the purified paraoxonase allozymes. How these particular amino acid substituents at position 191 influence the qualitative properties of the esterase must still be determined.

It is very likely that the serum enzyme is produced, at

Table I

Correlation of Serum Paraoxonase Phenotype and Genotype

Serum Type and Individual	PA[a] (μmol/min/ml)	PX[b] (μmol/min/ml)	PX/PA Ratio	Amino Acid 191	Amino Acid 54
A:					
Dli	95	155	1.6	Q, Q	M, M
Lst	100	169	1.7	Q, Q	M, M
Rso	100	191	1.9	Q, Q	M, M
Svo	82	152	1.9	Q, Q	M, M
Spr	98	169	1.7	Q, Q	M, L
Jko	62	105	1.7	Q, Q	M, L
Aha	122	212	1.7	Q, Q	M, L
Wwe	89	157	1.8	Q, Q	M, L
Sla	134	223	1.7	Q, Q	L, L
Dla	86	162	1.9	Q, Q	L, L
Pca	129	201	1.6	Q, Q	L, L
Ela	118	176	1.5	Q, Q	L, L
AB:					
Sad	58	321	5.6	Q, R	M, M
Kdi	100	627	6.3	Q, R	M, M
Bda	81	496	6.1	Q, R	M, L
Jde	141	784	5.6	Q, R	M, L
Tin	57	398	7.0	Q, R	M, L
Hca	51	355	7.0	Q, R	M, L
Kch	114	496	4.3	Q, R	L, L
Lze	129	758	5.9	Q, R	L, L
Mge	110	780	7.1	Q, R	L, L
B:					
Msm	91	846	9.3	R, R	L, L
Bla	86	890	10.3	R, R	L, L
Ata	106	1,006	9.5	R, R	L, L
Jda	76	737	9.7	R, R	L, L
Aca	100	1,063	10.7	R, R	L, L
Jva	106	1,020	9.7	R, R	L, L

[a] Phenylacetate.
[b] Paraoxon in the presence of 1 M NaCl.

least in part, by the liver. The serum esterase amino acid sequence determined by sequencing tryptic and peptic digest peptides has been found to be the same as that deduced from the human liver cDNA clones. Southern blots of human genomic DNA support the notion that there is a single gene for paraoxonase (Hassett et al. 1991). These authors also noted the two polymorphic sites and suggested that one or possibly both might be responsible for the polymorphic forms of the serum esterase.

It should be mentioned that in the tryptic analysis experiments extending over 10 years in our laboratory, with many preparations of purified enzyme preparations, we have seen only a few peptides that could not later be accounted for within the esterase sequence shown in figure 1. Most of these exceptions were fragments of apolipoprotein A-I. In addition, sequencing the undigested enzyme gave a strong, single peptide. This finding supports the notion that one enzyme has both paraoxonase and arylesterase activities. Thus, there is no evidence, from peptide or protein sequencing, for the presence of another protein copurifying with paraoxonase/arylesterase, and there is no supporting evidence for the proposal, by others, that human serum contains distinctly different enzymes for its paraoxonase and arylesterase activities (Mackness et al. 1987). Expression studies are in progress in our laboratory, to determine (1) whether enzymes produced from the cDNAs corresponding to the A and B phenotypes have the expected phenotypic properties and (2) whether they are able to hydrolyze both organophosphate and carboxylic acid esters.

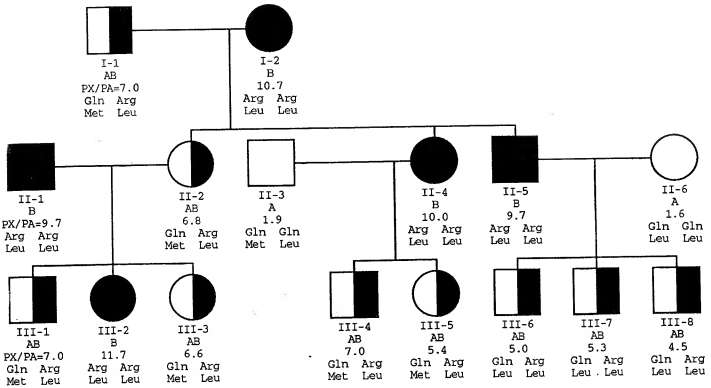

Figure 4 Comparison of human serum paraoxonase phenotype with Gln/Arg and Met/Leu polymorphisms as determined by sequencing of amplified genomic DNA. Unblackened symbols denote A-type individuals; half-blackened symbols denote AB-type individuals; blackened symbols denote B individuals; and PX/PA signifies the following ratio: nanomoles of paraoxon hydrolyzed (per minute per milliliter in the presence of 1 M NaCl at pH 10.5), divided by micromoles of phenylacetate hydrolyzed (per minute per milliliter) used to determine serum phenotype. The disposition of individual alleles in respect to Gln/Arg and Met/Leu polymorphism is shown.

To date, the number of individuals tested for the two polymorphic frequencies and linkage is too small to allow allelic frequencies to be calculated with any degree of confidence. However, it is clear that both polymorphisms are common ones, and, among the unrelated 27 individuals, Met had a frequency of .4 and Leu had a frequency of .6, at amino acid position 54. Met and Leu at position 54 were equally frequent in individuals homozygous for Gln at position 191 (A-type individuals), but Met 54 was not found in any individuals homozygous for Arg at position 191 (B-type individuals). Two AB-type individuals were homozygous for Met 54, so there were at least two Arg191/Met54 allozymic combinations represented in this small group. The total distance between these markers cannot be estimated accurately at this time, because the number and length of introns between these markers are still uncertain. However, the distance must be relatively short, and the appreciable linkage disequilibrium observed was not unexpected. The gene frequencies for the A/B polymorphism in different ethnic groups around the world have recently been reviewed (La Du 1992). The frequencies in Caucasian populations are about .71 and .29 for the A and B alleles, respectively. The somewhat lower frequency (.61) of the A (Gln) allele in our sample of 27 unrelated individuals is probably due to the small sample size. The presence of additional alleles affecting this esterase can be anticipated in the future. For example, more than one high-paraoxonase-activity allele has been proposed by Nielsen et al. (1986). A DNA basis for identification of such allelic variations will now be possible.

Note added in proof.—Similar conclusions about the molecular basis of the paraoxonase polymorphism have been reached by another laboratory, and these are described in a paper by Humbert et al. (in press).

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References

Bause E (1983) Structural requirements of N-glycosylation of proteins: studies with proline peptides as conformational probes. Biochem J 209:331–336

Black SD, Coon MJ (1982) Simple, rapid, and highly efficient separation of amino acid phenylthiohydantoins by reversed-phase high-performance liquid chromatography. Anal Biochem 121:281–285

Eckerson HW, Romson J, Wyte C, La Du BN (1983*a*) The human serum paraoxonase polymorphism: identification of phenotypes by their response to salts. Am J Hum Genet 35:214–227

Eckerson HW, Wyte CM, La Du BN (1983*b*) The human serum paraoxonase/arylesterase polymorphism. Am J Hum Genet 35:1126–1138

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6–13

Furlong CE, Richter RJ, Chapline C, Crabb JW (1991) Purification of rabbit and human serum paraoxonase. Biochemistry 30:10133–10140

Furlong CE, Richter RJ, Seidel SL, Motulsky AG (1988) Role of genetic polymorphism of human plasma paraoxonase/arylesterase in hydrolysis of the insecticide metabolites chlorpyrifos oxon and paraoxon. Am J Hum Genet 43:230–238

Gan KN, Smolen A, Eckerson HW, La Du BN (1991) Purification of human serum paraoxonase/arylesterase: evidence for one esterase catalyzing both activities. Drug Metab Dispos 19:100–106

Hamilton BA, Palazzolo MJ, Meyerowitz EM (1991) Rapid isolation of long cDNA clones from existing libraries. Nucleic Acids Res 19:1951–1952

Hassett C, Richter RJ, Humbert R, Chapline C, Crabb JW, Omiecinski CJ, Furlong CE (1991) Characterization of cDNA clones encoding rabbit and human serum paraoxonase: the mature protein retains its signal sequence. Biochemistry 30:10141–10149

Humbert R, Adler DA, Disteche CM, Hassett C, Omiecinski CJ, Furlong CE. The molecular basis of the human serum paraoxonase activity polymorphism. Nature Genet (in press)

Kwok SCM, Ledley FD, DiLella AG, Robson KJH, Woo SLC (1985) Nucleotide sequence of a full-length complementary DNA clone and amino acid sequence of human phenylalanine hydroxylase. Biochemistry 24:556–561

La Du BN (1992) Human serum paraoxonase/arylesterase. In: Kalow W (ed) Pharmacogenetics of drug metabolism. Pergamon, New York, pp 51–91

Lathe R (1985) Synthetic oligonucleotide probes deduced from amino acid sequence data: theoretical and practical considerations. J Mol Biol 183:1–12

Lockridge O, Bartels CF, Vaughan TA, Wong CK, Norton SE, Johnson LL (1987) Complete amino acid sequence of human serum cholinesterase. J Biol Chem 262:549–557

Mackness MI, Thompson HM, Hardy AR, Walker CH (1987) Distinction between 'A'-esterases and arylesterases. Biochem J 245:293–296

McGuire MC, Nogueira CP, Bartels CF, Lightstone H, Hajra A, Van der Spek AF, Lockridge O, et al (1989) Identification of the structural mutation responsible for the dibucaine-resistant (atypical) variant form of human serum cholinesterase. Proc Natl Acad Sci USA 86:953–957

Maniatis T, Fritsch EF, Sambrook J (1982) The identification of recombinant clones. In: Molecular cloning: a laboratory manual, 8th ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp 320–331

Mercer JFB, McAdams W, Chambers W, Walker ID (1986) The W and L allelic forms of phenylalanine hydroxylase in the rat differ by a threonine to isoleucine substitution. Biochem J 236:679–683

Mullenbach R, Lagoda PJL, Welter C (1989) An efficient salt-chloroform extraction of DNA from blood and tissues. Trends Genet 5:391

Playfer JR, Eze LC, Bullen MF, Evans DA (1976) Genetic polymorphism and interethnic variability of plasma paraoxonase activity. J Med Genet 13:337–342

Sanger F, Coulson AR, Barrell BJ, Smith AJF, Roe BA (1980) Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. J Mol Biol 13:161–178

Smolen A, Eckerson HW, Gan KN, Hailat N, La Du BN (1991) Characteristics of the genetically determined allozymic forms of human serum paraoxonase/arylesterase. Drug Metab Dispos 19:107–112

Tarr GE (1982) Manual batchwise sequencing methods. In: Elzinga M (ed) Methods in protein sequence analysis. Humana, Clifton, NJ, pp 223–232

Tsui LC, Buchwald M, Barker D, Braman JC, Knowlton R, Schumm JW, Eiberg H, et al (1985) Cystic fibrosis locus defined by a genetically linked polymorphic DNA marker. Science 230:1054–1057

EXHIBIT 6

The molecular basis of the human serum paraoxonase activity polymorphism

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The organophosphate cholinesterase inhibitor paraoxon is hydrolysed by serum paraoxonase/arylesterase. A genetic polymorphism of paraoxonase (PON) activity which determines high versus low paraoxon hydrolysis in human populations, may determine sensitivity to parathion poisoning. We demonstrate that arginine at position 192 specifies high activity PON whereas a glutamine specifies the low activity variant. Allele-specific probes or restriction enzyme analysis of amplified DNA allow for the genotyping of individuals. PON maps to chromosome 7q21–22, proximal to the cystic fibrosis gene, in agreement with previous genetic linkage studies.

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The metabolism of organophosphates, such as the insecticide parathion, has assumed importance since these toxicants came into widespread use over 40 years ago. Parathion is converted to its active species, paraoxon[1], via oxidative desulphuration by the microsomal cytochrome P450 system of liver and by other tissues[2–4]. Hydrolysis of paraoxon is catalysed by serum paraoxonase (PON)/arylesterase[5], an enzyme that is associated with the lipoprotein fraction of serum[6].

There is a 10–40-fold difference in serum PON activity between individuals[7–11]. For a given individual, PON activity is stable over time[9] and is genetically determined[12]. Populations of European ancestry[7–17] have approximately 50% homozygotes for an allozyme with low PON activity, 10% homozygotes for a high activity allozyme, and 40% heterozygotes. Some non-Caucasian populations show a reduced frequency of the low activity allele[11,17]. The differences observed between allelic classes are not simply quantitative; the high activity allozyme is stimulated more by NaCl[13,18,19] and has a higher pH optimum for maximal salt stimulation than the low activity allozyme[19]. The substrate-dependent activity polymorphism is also observed with methyl paraoxon, chlorthion-oxon, EPN-oxon, and armin (ethyl-p-nitrophenyl-ethylphosphonate)[19,20], but not with the substrates chlorpyrifos oxon, phenyl acetate, or β-naphthyl acetate[10,19].

Animal studies, including examination of the quantitative adequacy of PON in protection against paraoxon toxicity[21]; correlation of LD_{50} values with PON[22] and chlorpyrifos oxonase levels[23,24] and demonstration that intravenously injected PON provides protection against paraoxon and chlorpyrifos oxon toxicity[25,26], indicate that serum PON is protective against organophosphate poisoning. The large difference in turnover number between the allozyme types[19] suggests that humans may differ in susceptibility to parathion poisoning depending on PON allelic status, however differences in the amount of enzyme activity between individuals within each allelic class blur this distinction. Plots of chlorpyrifos oxon hydrolysis, or phenyl acetate hydrolysis[10,18] versus PON activity allow better discrimination between allelic classes.

In addition to its intrinsic interest, the PON activity polymorphism is of historical importance as it was the first genetic marker which was found to be linked to the cystic fibrosis (CF) gene[27,28]. In these early studies, Eiberg and collaborators used biochemical assays to determine that PON was linked to CF[27]. Subsequent studies linked both CF and *PON* to markers in the middle of the long arm of chromosome 7 (refs 29–34), which led to identification and positional cloning of the defective CF gene[35–38].

Recently we reported the isolation and sequencing of PON/arylesterase cDNAs from humans and rabbits[39]. Three independent human clones were isolated from a library constructed from a single human liver. The clones differed in (i) the predicted amino acid sequence at two positions, (ii) the extent of N-terminal sequence, and (iii) the site at which polyadenylation occurred. We have now examined the amino acid substitutions observed in the cDNA clones to account for the difference between the high and low allozyme types.

Characteristics of human cDNA clones

The inferred amino acid at position 55 of PON was methionine in clones HuPON1 and HuPON2 and leu-

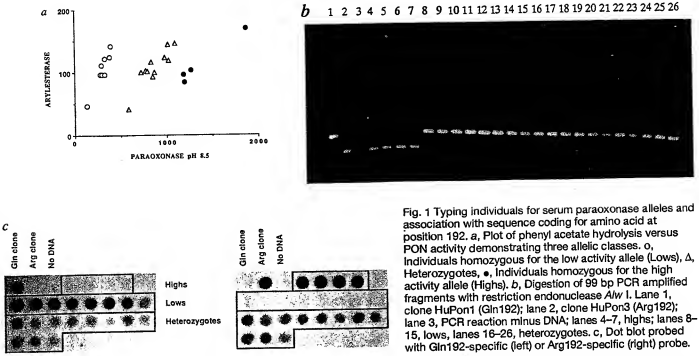

Fig. 1 Typing individuals for serum paraoxonase alleles and association with sequence coding for amino acid at position 192. *a*, Plot of phenyl acetate hydrolysis versus PON activity demonstrating three allelic classes. o, Individuals homozygous for the low activity allele (Lows), Δ, Heterozygotes, •, Individuals homozygous for the high activity allele (Highs). *b*, Digestion of 99 bp PCR amplified fragments with restriction endonuclease *Alw* I. Lane 1, clone HuPon1 (Gln192); lane 2, clone HuPon3 (Arg192); lane 3, PCR reaction minus DNA; lanes 4–7, highs; lanes 8–15, lows, lanes 16–26, heterozygotes. c, Dot blot probed with Gln192-specific (left) or Arg192-specific (right) probe.

in clone HuPON3; position 192 was glutamine in clones HuPON1 and HuPON2 and arginine in clone HuPON3[39]. Since heterozygotes for PON activity are abundant in the Caucasian population from which the cDNA library was prepared, we investigated whether either of the two observed amino acid substitutions was related to the activity polymorphism. The sequence differences noted in the cDNA clones are distinguishable by a change in a site for restriction endonuclease *Alw*I for the sequence corresponding to amino acid position 192 and a change in a *Nla*III site at position 55.

PON polymorphism and amino acid 192

In order to determine whether the observed amino acid difference(s) constituted the molecular basis of the activity polymorphism, we determined the phenotype of 23 individuals by enzyme assays[15]. The subjects fell into the expected three classes (that is homozygous low, heterozygous, and homozygous high; Fig. 1*a*). Polymerase chain reaction (PCR) fragments amplified from genomic DNA from each were examined for presence of the Gln192 versus Arg192 alleles by restriction analysis (Fig. 1*b*) and hybridization of allele-specific oligonucleotides to dot blots (Fig. 1*c*).

Four individuals predicted to be homozygous for the high activity allele from enzyme assays had amplified fragments which were cut by *Alw*I and which hybridized with the Arg192-specific oligonucleotide. Eight individuals homozygous for the low activity allele had PCR-amplified fragments which were not cut by *Alw*I and which hybridized with the Gln192-specific oligonucleotide. The PCR amplified segments from 11 heterozygotes were partially cut by *Alw*I and hybridized to both oligonucleotides. These results show that arginine is present in the high activity allozyme and glutamine is present in the low activity allozyme at position 192 of serum PON.

N-terminal region of *PON*

Clone HuPON3 is an incomplete reverse transcript which lacks the nucleotide sequence corresponding to the first 29 amino acids of the high acitivty serum PON/arylesterase. In order to eliminate the possibility that additional amino acid differences between the high and low activity alleles are present in this region, the DNA sequences for the first two coding exons corresponding to the first 48 amino acids of PON were amplified from genomic DNA and the sequences determined. DNA from three individuals homozygous for the high activity allele and four individuals homozygous for the low activity allele was analysed. The coding sequence for this region of all seven individuals was identical to the sequence of clone HuPON1. Additionally, we previously purified human PON from an individual homozygous for the high activity allele and determined by direct protein sequencing that the first 10 amino acids beyond the cleaved N-terminal methionine are identical to that found in the low activity clone HuPON1[40]. Thus, there are no differences between the high and low allozymes in the N-terminal region.

PON polymorphic site at position 55

We also examined the role of the amino acid at position 55. *Nla*III digests of PCR amplified fragments and oligonucleotide-specific dot blotting indicated that position 55 was polymorphic with an allele frequency of 0.74 for the leu55 allele and 0.26 for the met55 allele in our sample (data not shown). Members of the homozygous low activity group were found with all three possible combinations of position 55 alleles (Met55/Met55, Met55/Leu55, and Leu55/Leu55). The lack of concordance between allelic status of the activity polymorphism and residue 55 indicates that this position is not involved in determination of the allozyme type.

Mapping human *PON* cDNA clones

Somatic cell hybrid and restriction fragment length polymorphism studies have mapped the biochemically defined *PON* locus to the middle of the long arm of chromosome 7 near the CFTR locus[32–34]. We used *in situ* hybridization of radiolabelled cDNA to human metaphase

chromosomes to determine whether the clone hybridized to the predicted location. We found that autoradiographic grains were localized to bands q21 and q22 on chromosome 7 (Fig. 2), as expected. The lack of significant hybridization to other chromosomes confirms our earlier conclusions based on Southern blot analysis that serum PON is probably not a member of a multi-gene family[20].

Discussion

The PON activity polymorphism has been the subject of numerous studies for more than 20 years[20]. Our results indicate that the difference between the two allozyme types is due to a single amino acid substitution which can easily be determined by restriction enzyme analysis. These two polymorphic markers will be useful in future genetic and physical mapping studies of Chromosome 7 — our *in situ* hybridization data confirm the earlier chromosomal assignments of *PON* based on the activity polymorphism.

Substitution of glutamine for arginine at position 192 affects the charge of the enzyme and might be expected to have effects on substrate turnover number if near the active site. The presence of Arg192 in the high activity allozyme in humans compares to a Lys192 residue in rabbits. The rabbit enzyme also has a high turnover number for paraoxon.

The high level of conservation (>85%) between human and rabbit serum PON sequences[31] suggests a conserved function. PON activity has been reported to be reduced following myocardial infarction[41]. Addition of purified PON was found to reduce oxidation of LDL[42]. The low activity allele has been associated with lower triglyceride and higher LDL cholesterol[43], however the anomalously small number of homozygous high individuals suggests that some misclassification of subjects may have occurred. Biochemical assays do not easily distinguish heterozygotes at the high end of the range from homozygotes for the high allele. The molecular assays described here provide accurate discrimination between these groups. By allowing accurate typing of subjects, any physiological effects of the PON activity polymorphism will be more easily measured in future work. The studies of association with lipid levels[41–43] and localization to the HDL particle[6] suggest PON involvement in lipid metabolism, but as yet no physiological substrate has been identified. It is thus not yet possible to define the importance of the PON activity polymorphism in normal metabolism. The animal studies noted above have established that serum PON is important in protection against toxicity due to organophosphate substrates of the enzyme.

Methodology

Human subjects. 23 subjects were investigated, including five subjects whose allelic status was known from previous biochemical studies (two were homozygous for the low allele, one was heterozygous, and two were homozygous for the high activity allele). 22 subjects were of Caucasian ancestry and one was of mixed Caucasian and Chinese ancestry. Blood samples for assay of PON activity were collected in lithium-heparin tubes. Blood samples for DNA preparation were collected in EDTA tubes.

Lymphocyte DNA. Lymphocytes were isolated from blood and DNA was prepared as described[44].

Enzyme assays. Hydrolysis of phenyl acetate[10] and paraoxon[24] were measured as described.

Amplification of DNA. Sequence information from genomic clones encoding serum PON (R.H. and C.E.F., unpublished data) allowed design of primers for PCR amplification[45] of specific segments of PON genomic DNA from human subjects. All primers were used at 0.5 μM. An initial incubation of 3 min at 93–95 °C preceded amplification of genomic DNA. Primers for amplification of a 99 bp sequence coding for position 192 were 5'TATTGT TGCTGTGGGACCTGAG3' and 5'CACGCTAAACCCAAATAC-ATCTC3'. Primary amplification (30 cycles) was performed using Amplitaq polymerase (Perkin Elmer–Cetus) in 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-Cl pH 8.3 for cycles of 60 s at 93 °C, 30 s at 61 °C, and 60 s at 72 °C. DNA from a 16 cycle secondary amplification was used for digests and dot blots.

Primers for amplification of position 55 were 5'GAAGAGTGA-TGTATAGCCCCAG3' and 5'TTTAATCCAGAGCTAATGAA-AGCC3'. Amplification for 35 cycles of 94 °C for 30 s, 61 °C for 30s, and 72 °C for 30 s produced a 170 bp amplified product.

Sequence missing from clone HuPON3 is encoded by 2 exons in human genomic DNA. Primers for amplification of the sequence corresponding to the amino terminus of the serum PON protein from the first coding exon (24 2/3 codons) were 5'GCAGCAGAGCCTCCTAGC3' and 5'GCACCTGCTTGTAAAT-GTTCTG3'. Primers for amplification of the second coding exon (23 1/3 codons) were 5'TCTGGAGTTGAAACTCAGGC3' and 5'TGGACAGATTGAACAGGCAC3'. Amplification was performed with Vent polymerase (New England Biolabs) in the recommended buffer with 30 cycles of 120 s at 95 °C, 120 s at 53 °C, and 60 s at 78 °C.

DNA sequencing. Amplified fragments were isolated from agarose gels, purified using glass fines[46], and sequenced using ^{32}P end labelled primers and *Taq* polymerase with a Cycle sequencing kit (Gibco-BRL) using the recommended conditions.

Allele specific oligonucleotides for determination of genotype. For dot blots, 1 μl amplified DNA was applied to nitrocellulose filters which were incubated 2 × 5 min in 0.5 N NaOH, 1.5 M NaCl, 2 × 5 min in 1 M Tris-Cl pH 7.4, 1.5 M NaCl. Filters were exposed to a UV transilluminator for 2 min, then dried. Following prehybridization for 60 min in the indicated hybridization buffer, filters were incubated with Gln192-specific (5'CCTACTTACAAATCCTCGGGA3'), or Arg192-specific (5'CCTACTTACGATCCTCGGGA3'), end-labelled probes for 90 min at 55 °C and washed for 5 min at 22 °C and 5 min at 40 °C. For the Gln192 probe, incubation was in 5× SSPE, 0.5% SDS, 0.1% ficoll, 0.1% polyvinyl pyrrolidone, 0.1% BSA, 100 μg ml^{-1} salmon sperm DNA; washes were with 2× SSC, 0.5% SDS. For

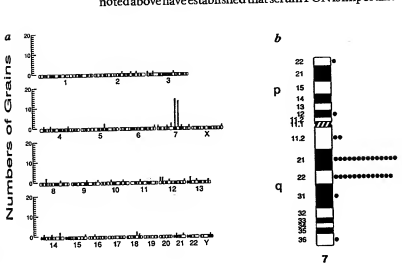

Fig. 2 Mapping the PON gene by *in situ* hybridization. *a*, Distribution of autoradiographic grains on human chromosomes from 49 metaphase cells. 37% of a total of 73 sites of hybridization are on chromosome 7 at bands q21 and q22. There is no significant hybridization to other chromosomes. *b*, Localization of autoradiographic grains to bands q21 and q22 of chromosome 7.

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the Arg192 probe, incubation was in 3× SSPE, 0.5% SDS, 0.1% ficoll, 0.1% polyvinyl pyrrolidone, 0.1% BSA, 100 μg ml^{-1} salmon sperm DNA; washes were with 1× SSC, 0.5% SDS (1× SSC is 0.15 M NaCl, 0.015 M Na citrate, pH 7.0; 1× SSPE is 0.15 M NaCl, 0.01 M NaH_2PO_4, 1 mM ethylene diaminetetraacetic acid, pH 7.4).

Digestion of amplified fragments with restriction endonucleases. Amplified fragments were precipitated with ethanol and resuspended in buffer recommended by the manufacturer. 5 μl of amplified product was digested with 2 U of enzyme (*AlwI* for analysis of position 192 or *NlaIII* for analysis of position 55) for 2–3 h at 37 °C. The products were analysed on a 3% NuSieve (FMC corp.) agarose gel.

***In situ* hybridization** of cDNA to human chromosomes. A clone HuPONI containing a 1346 basepair *EcoRI* fragment was radiolabelled with [^{3}H]-labelled nucleotides by nick translation to 3 × 10^7 cpm μg^{-1}. The probe at a concentration of 0.005 to 0.01 ng ml^{-1} was hybridized *in situ* to metaphase chromosomes from human lymphocytes, as described[47].

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1. Gage, J.C. A cholinesterase inhibitor derived from O,O-diethyl-O-p-nitrophenyl thiophosphate *in vivo*. *Biochem. J.* 54, 426–430 (1954).
2. Diggle, W. M. & Gage, J. C. Cholinesterase inhibition by parathion *in vivo*. *Nature* 168, 996 (1951).
3. Davidson, A. N. The conversion of schradan (OMPA) and parathion into inhibitors of cholinesterase by mammalian liver. *Biochem. J.* 61, 203–209 (1955).
4. Poore, R. E. & Neal, R. A. Evidence for extrahepatic metabolism of parathion. *Toxicol. Appl. Pharmacol.* 23, 759–768 (1972).
5. Aldridge, W. N. An enzyme hydrolysing diethyl p-nitrophenyl phosphate (E 600) and its identity with the A-esterase of mammalian sera. *Biochem. J.* 53, 117–124 (1953).
6. Mackness, M. I. & Walker, C. H. Partial purification and properties of sheep serum 'A' esterases. *Biochem. Pharmacol.* 32, 2291–2296 (1983).
7. Krisch, K. Enzymatische hydrolyse von Diäthyl-p-nitrophenylphosphat (E 600) durch menschliches serum. *Z. klin. Chem. u. klin. Biochem.* 5, 41–45 (1967).
8. Geldmecher-von Mallinckrodt, M., Rabast, U. & Lindorf, H. H. Untersscheidliche reaktion menschlicher serum-cholinesterasen mit E600 (O,O-diäthyl-O-(p-nitrophenyl)-phosphat. *Arch. Toxikol.* 25, 223–228 (1969).
9. Zech, R. & Zurcher, K. Organophosphate splitting enzymes in different mammals. *Comp. Biochem. Physiol.* 48B, 427–433 (1974).
10. Furlong, C. E., Richter, R. J., Seidel, S. J. & Motulsky, A. Role of genetic polymorphism of human plasma paraoxonase/arylesterase in hydrolysis of the insecticide metabolites chlorpyrifos oxon and paraoxon. *Am. J. hum. Genet.* 43, 230–238 (1988).
11. Playfer, J. R., Eze, L. C., Bulen, M. F. & Evans, D. A. P. Genetic polymorphism and interethnic variability of plasma paraoxonase activity. *J. med. Genet.* 13, 337–342 (1976).
12. Geldmacher-von Mallinckrodt, M. *et al.* Genetisch determinierter polymorphismus der menschlichen serum-paraoxonase (EC 3.1.1.2) *Humangenetik* 17, 331–335 (1973).
13. Mueller, R. F. *et al.* Plasma paraoxonase polymorphism: a new enzyme assay, population, family, biochemical, and linkage studies. *Am. J. Hum. Genet.* 35, 393–408 (1983).
14. Eiberg, H. & Mohr, J. Genetics of paraoxonase. *Ann. hum. Genet.* 45, 323–330 (1981).
15. Eckerson, H. W., Whyte, C. M. & LaDu, B. N. The human serum paraoxonase/arylesterase polymorphism. *Am. J. hum. Genet.* 35, 1126–1138 (1983).
16. Ortigoza-Ferado, J., Richter, R. J., Hornung, S., Motulsky, A. G. & Furlong, C. E. Paraoxon hydrolysis in human serum albumin mediated by a genetically variable arylesterase and albumin. *Am. J. hum. Genet.* 36, 295–305 (1984).
17. Geldmecher-von Mallinckrodt, M. & Diepgen, T. L. The polymorphism of the human serum paraoxonase. *Toxicol. Environ. Chem.* 14, 165–181 (1987).
18. Eckerson, H. W., Romson, J., Whyte, C. & LaDu, B. The human serum paraoxonase polymorphism: identification of phenotypes by their response to salts. *Am. J. hum. Genet.* 35, 214–227 (1983).
19. Smolen, A., Eckerson, H. W., Gan, K. N., Hallat, N. & LaDu, B. N. Characteristics of the genetically determined allozymic forms of human serum paraoxonase/arylesterase. *Drug Metab. Dispos.* 19, 107–112 (1991).
20. Geldmacher-von Mallinckrodt, M. & Diepgen, T. L. The human serum paraoxonase - polymorphism and specificity. *Toxicol. Environ. Chem.* 18, 79–196 (1988).
21. Butler, E. G., Eckerson, H. W. & LaDu, B. N. Paraoxon hydrolysis vs. covalent binding in the elimination of paraoxon in the rabbit. *Drug Metab. Dispos.* 13, 640–645 (1985).
22. Costa, L. G. *et al.* Species differences in serum paraoxonase correlate with sensitivity to paraoxon toxicity. In *Toxicology of Pesticides: Experimental, Clinical and Regulatory Aspects* (ed. Costa, L. G.) 263–266 (Springer, Berlin, 1987).
23. McCollister, S., Kociba, R. J., Humiston, C. G., McCollister, D. D. & Gehring, P. J. Studies of the acute and long-term oral toxicity of chlorpyrifos (O,O-diethyl-O-[3,5,6-trichloro-2-pyridyl] phosphorothioate) *Cosmet. Toxicol.* 12, 45–61 (1974).
24. Furlong, C. E. *et al.* Spectrophotometric assays for the enzymatic hydrolysis of the active metabolites of chlorpyrifos and parathion by plasma paraoxonase/arylesterase. *Anal. Biochem.* 180, 242–247 (1989).
25. Main, A. R. The role of A-esterase in the acute toxicity of paraoxon, TEPP, and parathion. *Canad. J. Biochem. Physiol.* 34, 197–216 (1956).
26. Costa, L. G. *et al.* Serum paraoxonase and its influence on paraoxon and chlorpyrifos oxon toxicity in rats. *Toxicol. Appl. Pharmacol.* 103, 66–76 (1990).
27. Eiberg, H., Mohr, J., Schmiegelow, K., Nielsen, L. S. & Williamson, R. Linkage relationships of paraoxonase (PON) with other markers: indication of PON-cystic fibrosis syntany. *Clin. Genet.* 29, 265–271 (1986).
28. Schmiegelow, K. *et al.* Linkage between the loci for cystic fibrosis and paraoxonase. *Clin. Genet.* 29, 374–377 (1986).
29. Knowlton, R. G. *et al.* A polymorphic DNA marker linked to cystic fibrosis is located on chromosome 7. *Nature* 318, 380–382 (1985).
30. White, R. *et al.* A closely linked genetic marker for cystic fibrosis. *Nature* 318, 382–384 (1985).
31. Wainwright, B. J. *et al.* Localization of cystic fibrosis to human chromosome 7cen-q22. *Nature* 318, 384–385 (1985).
32. Rommens, J. M. *et al.* Identification and regional localization of DNA markers on chromosome 7 for the cloning of the cystic fibrosis gene. *Am. J. hum. Genet.* 43, 645–663 (1988).
33. Beaudet, A. *et al.* Linkage of cystic fibrosis to two tightly linked DNA markers: joint report from a collaborative study. *Am. J. hum. Genet.* 39, 681–693 (1986).
34. Tsui, L.-C. *et al.* Genetic analysis of cystic fibrosis using linked DNA markers. *Am. J. hum. Genet.* 39, 720–728 (1986).
35. Rommens, J. M. *et al.* Identification of the cystic fibrosis gene: chromosome walking and jumping. *Science* 245, 1059–1066 (1989).
36. Riordan, J. R. *et al.* Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science* 245, 1066–1073 (1989).
37. Kerem, B.-S. *et al.* Identification of the cystic fibrosis gene: genetic analysis. *Science* 245, 1073–1080 (1989).
38. Collins, F. S. Cystic fibrosis: molecular biology and therapeutic implications. *Science* 256, 774–779 (1992).
39. Hassett, C. *et al.* Characterization of cDNA clones encoding rabbit and human serum paraoxonase. Biochemistry 30, 10133–10140 (1991).
40. McElveen, J. *et al.* Distribution of paraoxon hydrolytic activity in the serum of patients following myocardial infarction. *Clin. Chem.* 32, 671–673 (1986).
41. Mackness, M. I., Arrol, S. & Durrington, P. N. Paraoxonase prevents accumulation of lipoperoxides in low-density lipoprotein. *FEBS Lett.* 286, 152–154 (1991).
42. Saha, N., Roy, A. C., Teo, S. H., Tay, J. S. H. & Ratnam, S. S. Influence of serum paraoxonase polymorphism on serum lipids and apolipoproteins. *Clin. Genet.* 40, 277–282 (1991).
43. Poncz, M. *et al.* Construction of human gene libraries from small amounts of peripheral blood. Analysis of β-like globin genes. *Hemoglobin* 6, 27–36 (1982).
44. Saiki, R. K. *et al.* Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239, 487–491 (1988).
45. Vogelstein, B. & Gillespie, D. Preparative and analytical purification of DNA from agarose. *Proc. natn. Acad. Sci. U.S.A.* 76, 615–619 (1979).
46. Marth, J. D. *et al.* Localization of a lymphocyte-specific protein tyrosine kinase gene (lck) at a site of frequent chromosomal abnormalities in human lymphomas. *Proc. natn. Acad. Sci. U.S.A.* 83, 7400–7407 (1986).